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# Pulmonary inflammation induced by repeated inhalations of β(1,3)-D-glucan and endotoxin

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**Summary.** In an animal model of hypersensitivity pneumonitis (HP) guinea-pigs were exposed for 5 weeks to an aerosol of bacterial endotoxin, β(1,3)-D-glucan (curdlan) or a combination. Exposure to endotoxin or curdlan showed only small changes in inflammatory cells in airways or the lung wall, histologically or in terms of enzyme secretion from alveolar macrophages. When the two agents were given together, a histology resembling HP was seen with alveolar infiltrates and early granulomas. Inflammatory cells in airways were increased and enzyme production of macrophages was changed, suggesting an effect of curdlan on the inflammatory regulating capacity of airway macrophages. The results suggest that interference with macrophage function and inflammation are important components in the development of HP.

*Keywords*: hypersensitivity pneumonitis, β(1,3)-D-glucan, endotoxin, inflammation, macrophages

Exposure to organic dusts may lead to different inflammatory responses, among them a granulomatous inflammation named hypersensitivity pneumonitis (HP) (Richerson 1983). Organic dusts contain a mixture of different agents with biological potency such as fungi, bacteria, proteins and animal and plant constituents (Rylander & Peterson 1990) which alone or in combination could be the causative agents for this disease.

Specific substances in organic dusts with particularly important effects are bacterial endotoxins and β(1,3)-D-glucans. Endotoxins are lipopolysaccharide compounds on the outer cell wall of Gram-negative bacteria and cause an inflammatory response in the airways and the lung tissue after inhalation (Burrell 1990; Snella *et al.* 1987; Venaille *et al.* 1989).

β(1,3)-D-glucans are present in the cell wall of fungi and *Actinomyceter*. They are polyglucose compounds consisting of a glucose chain united by β(1,3)-D-linkages (di Luzio 1985). β(1,3)-D-glucans are potent inducers of inflammation as well as modulators of the immune system (Cook *et al.* 1980; di Luzio 1985; Fogelmark *et al.* 1992). β(1,3)-D-glucans can also produce granulomas (Cook *et al.* 1980; Johnson *et al.* 1984).

HP is a granulomatous inflammation in the lungs (Richerson 1983). The initial stage of the disease is a general inflammatory cell infiltrate in the airways and without evidence of granulomas or lung function decrease. This stage may be present in a high proportion of an exposed population (Larsson *et al.* 1988). In the later stages, the inflammatory cells present a picture of focal granulomatous inflammation in the parenchyma and with a predominance of mononuclear cells. A transient neutrophilia may be present in acute exacerbations of the disease, induced by high levels of exposure (Fournier *et al.* 1985). In the chronic stage, distinct granulomas and sometimes fibrosis may develop. The fully developed disease requires a certain time of exposure to develop (Schuyler *et al.* 1982).

An increased risk for HP has been related to environ-

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ments with exposure to dust from mouldy materials (Richerson 1983). In earlier experimental studies in animals, we have demonstrated that a pathology resembling early HP may be produced in guinea-pigs inhaling dust from mouldy hay (Fogelmark *et al.* 1989; Fogelmark & Rylander 1993) or different species of microorganisms such as moulds and *Actinomyceter* (Fogelmark *et al.* 1991). It can be hypothesized that the β(1,3)-D-glucan present in these contributes to the development of the acute and chronic inflammatory responses observed.

To evaluate the role of β(1,3)-D-glucan and to study the possible interference of bacterial endotoxins for the development of early HP, experiments were undertaken where animals were exposed to an aerosol of these agents separately and in combination during a 5-week period. The effects were evaluated as the histopathological changes in the lung tissue, the number of inflammatory cells in the airways and the lung interstitium and the amount of lysozomal enzymes in lung lavage fluid and in the cells from the same compartments.

## Materials and methods

### *Materials*

For the β(1,3)-D-glucan exposures, we used a water insoluble form (Curdlan, Waiko Pure Chemical Ind., Tokyo). The curdlan was suspended in distilled water (100 μg/ml). For the endotoxin exposures we used a purified lipopolysaccharide (*Escherichia coli* 026 B6, Difco Lab) dissolved in distilled water (10 μg/ml). An aerosol of the different solutions was generated by a Collison atomizer (Rylander 1968) and fed into an exposure chamber.

### *Animals*

Female guinea-pigs with an initial weight of 400–500 g were placed in exposure chambers for 4 hours daily and then kept in animal cages supplied with filtered air at slight over-pressure. Food and water were supplied *ad lib*. The animals were exposed for 4 hours a day, 5 days per week during 5 weeks. They were examined 24 hours after the last exposure.

### *Lung lavage cells*

After a lethal i.p. dose of pentothal, the lung vascular 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 counting the number of inflammatory cells in the lavage fluid, lung walls and for the enzyme assays. The left lung was used for histological examinations and its main bronchus was tied off before the lavage procedure.

The right lung was subject to lung lavage. A body weight of 400–500 g corresponded to 50 ml of saline and for each additional 100 g we increased the lavage volume by 10 ml. The saline was divided into 10 aliquots, slowly injected into the bronchus 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, counted and typed using May–Grünewald–Giemsa stain. These cells are referred to as lung lavage cells (LLC). The first portion of the lung lavage supernatant was saved for enzyme analysis.

### *Lung wall cells*

The right lung was prepared using a modification of a technique previously described (Holt *et al.* 1985; Snella *et al.* 1987). The lung was cut out 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 minutes 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. Viability was controlled by Trypan blue exclusion (the range was 80–92%). These cells are referred to as lung wall cells (LWC).

### *Enzyme assays*

The activity of enzymes was measured in lung lavage fluid and LLC and LWC lysed in 0.1% Triton-X-100. *N*-Acetyl-β-D-glucosaminidase (NAG) and cathepsin D were assayed according to previously described methods (Rylander *et al.* 1985). Enzyme activities were expressed in μmol/ml *p*-nitrophenol for *N*-acetyl-β-D-glucosaminidase and units/ml tyrosine for cathepsin D (all reagents from Sigma). Total protein was determined by the methods of Bio-Rad-Protein Assay (BioRad Laboratories) and expressed in mg total protein/ml. The enzyme data from cells were expressed as activity/mg protein.

### *Histology*

The left lung was removed and fixed by slowly injecting 4% buffered formaldehyde into the bronchus. After dehydration and embedding, the lung was sectioned

along the bronchus and stained with Weigert haematoxylin–eosin stain. The degrees of cell infiltrate and the presence of intra-alveolar cells and granulomas were determined and scored on a scale of 1–5, according to the following scheme:

1: normal lung
2: cell infiltration in alveoli, no interstitial cell infiltration and no alveolar wall thickening
3: severe cell infiltration in alveoli, interstitial cell infiltration and alveolar wall thickening
4: cell aggregation in alveoli, alveolar wall thickening with increased numbers of interstitial cells, granuloma formation
5: defined granulomas

The severity index for the histological changes in a group of animals was calculated as the mean of each animal score. The slides were read at random with no identification as to the exposure.

Among certain of the animals, exposed as well as controls, large well defined areas were found with a high cell density and a monotonous picture of mononuclear cells. In adjacent, normal lung tissue, no cell increases in the alveoli and no signs of early granuloma formation were found. We interpreted these changes as due to a previous infection or other insult to the lung. The histological evaluation was based only on animals not showing these changes.

### *Treatment of data*

The mean numbers of each cell type were calculated for each group of animals and the statistical significance of differences between groups was evaluated using Student's *t*-test. For the histological index, Fisher's exact test was used.

## Results

### *Lung lavage cells*

The numbers of LLC in animals from the different exposure experiments are shown in Figure 1.

Exposure to curdlan did not cause any significant change in any cell type in the lavage fluid although a tendency to a decrease in the number of lymphocytes was found. In animals exposed to endotoxin there was a significantly higher number of all four cell types (macrophages $P<0.001$, lymphocytes $P<0.01$, neutrophils $P<0.001$, eosinophils $P<0.001$) as compared to control animals.

In animals exposed to a combination of curdlan and endotoxin, the number of LLC types was also significantly elevated over the controls (macrophages $P<0.001$, lymphocytes $P<0.001$, neutrophils $P<0.001$ and eosinophils $P<0.001$). Compared to the group exposed to endotoxin alone, the number was significantly higher for macrophages, lymphocytes and neutrophils ($P<0.01$, 0.01 and 0.01 respectively).

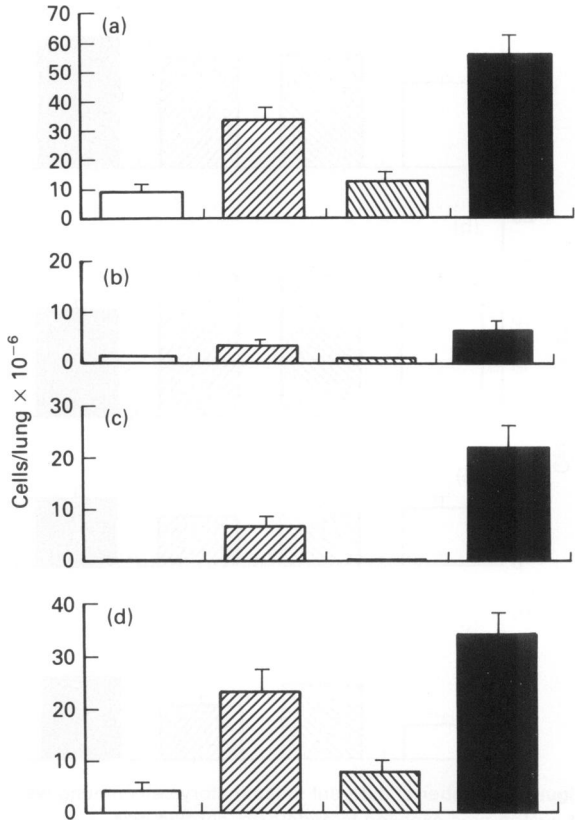


**Figure 1.** Number of different inflammatory cells in lung lavage of guinea-pigs exposed to endotoxin, curdlan or a combination of both. Mean values from 16 animals; bars indicate s.d. a, Macrophages; b, lymphocytes; c, neutrophils; d, eosinophils. □, Control; ▨, endotoxin; ▧, curdlan; ■, curdlan + endotoxin.

### *Lung wall cells*

The numbers of LWC are shown in Figure 2.

In animals exposed to curdlan, there were slight but non-significant increases in macrophages and eosinophils as compared to controls. In animals exposed to endotoxin, the number of eosinophils was increased ($P<0.001$). In animals exposed to the combination of curdlan and endotoxin, the number of neutrophils and eosinophils was slightly although not significantly increased above those exposed to endotoxin only. The number of lymphocytes was lower than in controls or

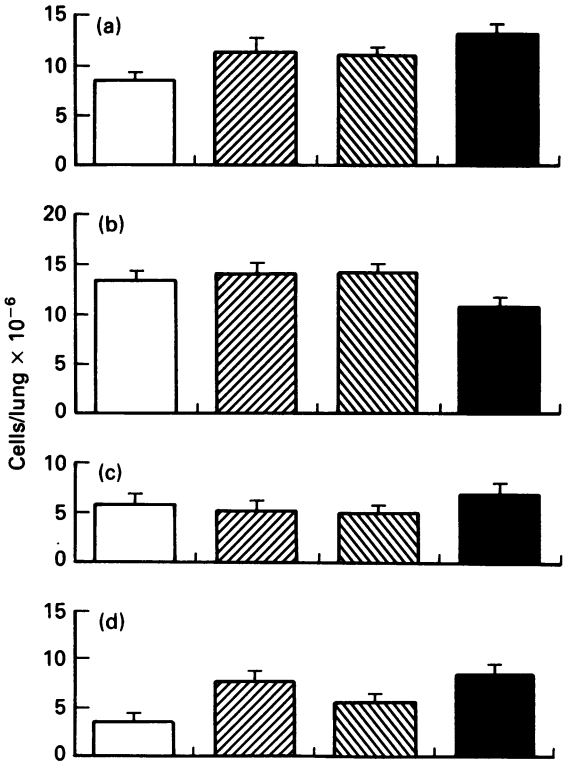


**Figure 2.** Number of different inflammatory cells in lung wall of guinea-pigs exposed to endotoxin, curdlan or a combination of both. Mean values from 16 animals; bars indicate s.d. a, Macrophages; b, lymphocytes; c, neutrophils; d, eosinophils. □, Control; ■, endotoxin; ▧, curdlan; ■, curdlan + endotoxin.

**Table 1.** Histological evaluation lungs from guinea-pigs exposed to curdlan, endotoxin or both. Animals with extended chronic infiltrates (see text) excluded (5 in controls and 3 in each of the other groups)

| Exposure | *n* | Average score | *n*>2 |
|---|---|---|---|
| Control | 11 | 1.6 | 0 |
| Curdlan | 13 | 1.7 | 1 |
| Endotoxin | 13 | 2.6 | 2 |
| Curdlan + endotoxin | 13 | 3.5 | 7 |

endotoxin exposed animals although the difference was not statistically significant.

*Histology*

The results from the histological evaluations are shown in Table 1.

In animals exposed to curdlan, no differences in the histological grading could be demonstrated as compared to controls. In animals exposed to endotoxin, a cell infiltrate in the lung was noticed, mainly as a cell invasion into the alveoli but sometimes also as an increased cell number in the alveolar walls resulting in a mean score of 2.6 (not statistically significant).

In animals exposed to a combination of curdlan and endotoxin, pronounced effects were found. The mean score was increased above those for endotoxin or curdlan exposed animals and the number of animals with a score higher than 2 was significantly increased ($P = 0.048$ and 0.005 compared to curdlan and endotoxin respectively, Fisher's exact test).

*Enzymes*

The enzyme production in lysed LLC and LWC are shown in Figure 3.

After endotoxin exposure, the concentrations of NAG and cathepsin D were increased in LLC ($P < 0.001$) and to a lesser extent in LWC ($P = 0.014$ and 0.059 respectively). The exposure to curdlan did not affect enzyme production. In animals exposed both to endotoxin and curdlan, the previously shown increase in the amount of these enzymes after endotoxin exposure was still present but in LLC it was significantly lower than after endotoxin exposure only ($P < 0.008$ and $< 0.006$ respectively).

In the lung lavage fluid, no significant differences in enzyme levels were found between the groups. There was a slight and similar increase in total protein in the groups exposed to LPS and to curdlan and endotoxin (data not shown).

## Discussion

The experimental model used comprises the exposure of the agents studied by inhalation only and in doses similar to those in the environment. It is thus closer to real conditions than previously used models, where preimmunization with injection of adjuvants or intratracheal injections of large doses of the agent studied have been used, to produce the granulomatous response in HP. The findings reported represent an early phase of HP with pregranulomatous changes; fibrosis was not detected. Fibrosis is however a rare complication in HP (Malmberg *et al.* 1988; Richerson 1983) and may require longer exposure times for development. In studies on inhaled silica particles, fibrosis was not detected till several months after the cessation of exposure (Sjöstrand & Rylander 1987).

Exposure to endotoxin caused an increase in cell numbers in the airways. The increase in neutrophils and other inflammatory cell types is the typical response after this exposure as demonstrated in previous studies (Snella *et al.* 1987; Venaille *et al.* 1989). In the lung wall the major response was an increase in the number of eosinophils. The increases in cell numbers in this as well

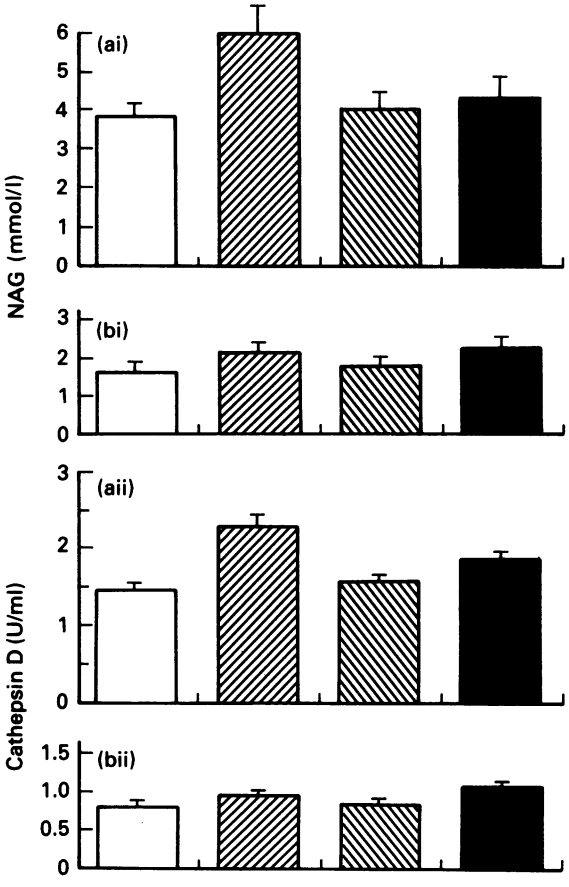


**Figure 3.** Amount of i, *N*-acetyl-β-D-glucosaminidase and ii, cathepsin D in lysed a, lung lavage cells and b, lung wall cells. Average values and s.d., 5 weeks exposure. □, Control; ▨, LPS; ▧, curdlan; ■, curdlan + endotoxin.

as in a previous long-term exposure study (Snella *et al.* 1982) were less than in acute exposure studies suggesting an adaptation. This could be due to the macrophages secreting less of chemotactic factors or producing a factor decreasing the migration of neutrophils.

The results from the enzyme analysis also suggest an adaptation after long-term exposure. In previous studies on acute effects after endotoxin exposure (Rylander *et al.* 1985), an increase in enzyme levels was found in lung lavage fluid and a lower value in lung lavage cells, suggesting acute damage with leakage of enzymes out of the cells. In this study, no signs of leakage could be found after endotoxin exposure. Instead, the production of enzymes was high in the lavage cells, indicating an effect of the exposure without damage to the cell walls. Small signs of cell damage were however found in that the protein content of the lavage fluid was higher than in unexposed, suggesting endothelial/epithelial cell damage.

In this study, no effects of the exposure to curdlan were detected. A previous acute inhalation study (Fogelmark *et al.* 1992) also showed that curdlan inhalation did not change the number of inflammatory cells in the lung. In both studies, there were indications that the number of lymphocytes was decreased but the changes were small.

The most striking finding was the synergistic effect of endotoxin and curdlan on the LLC and the histological changes with a large influx of inflammatory cells into the alveolar space and beginning of granuloma formation. The changes resemble those previously reported after repeated exposures to mouldy hay (Fogelmark *et al.* 1989) and from other models of HP (Schuyler *et al.* 1982). There was no increase of inflammatory cells in the lung wall, suggesting that the reaction produced by the combined exposure was mainly located in the airways. The combined exposure significantly decreased the production of NAG and cathepsin D in lavage cells, compared to animals exposed to endotoxin only. The discrepancy between enzyme production in lavage cells from endotoxin and glucan/endotoxin exposed animals is probably not caused by a shift in proportion of one or several cell types, since the maximum shift was 8% for neutrophils and 5% for macrophages. The influx of inflammatory cells in the combined exposure and the down regulating effect of cell lysosomal enzyme production, compared to endotoxin exposure alone, suggests that curdlan interfered with the function of the macrophages.

By contrast, the LWC show only minor changes. In studies where animals were exposed to silica dust, profound changes were found in LWC with increased number of low density macrophages which also showed increased enzyme production (Davis *et al.* 1978). One possible reason for this discrepancy is differences in particle characteristics between silica and curdlan. Different types of materials and different particle surface characteristics may cause the glucan particle not to penetrate the airway epithelium, causing lysosomal enzyme production in the LWC. The inflammatory response caused by endotoxin exposure may facilitate the transfer into the lung wall.

A synergistic effect has also been found in previous studies when endotoxin and β(1,3)-D-glucan have been given by injection. Depending on the type of animal model used the responses vary however from a suppression of macrophage function to an increased resistance to infection (Franek *et al.* 1992). It is likely that the seemingly divergent results are due to the different animal models used or to differences in the cellular dose level.

Regarding endotoxins, it has previously been shown

that curdlan exposure renders the animals more susceptible to Gram-negative sepsis or endotoxin exposure (Franek *et al.* 1992). Concerning the lung, a previous study reported that curdlan decreased the neutrophil invasion after an exposure to endotoxin (Fogelmark *et al.* 1992). It was suggested that this was due to a suppressive effect on macrophages.

There is general agreement that a major effect of glucan is on macrophages (Cook *et al.* 1980; di Luzio 1985; Johnson *et al.* 1984). The present findings suggest that the mechanism whereby inhaled glucan sensitizes animals to endotoxin is through a decrease in its normal function.

The findings in this study are of significance with relation to real life exposure conditions. HP is thought to develop after exposure to a large number of microorganisms (Malmberg *et al.* 1988; Richerson 1983; Rylander & Peterson 1990). Mouldy hay and other mouldy materials also contain Gram-negative bacteria and considerable amounts of endotoxin can thus be present. In this perspective, the results from this study suggest that the development of HP is a process requiring an exposure to at least two agents, one with an effect on the function of macrophages with a secondary interference with lymphocytes, and the other with the capability to cause a traditional inflammatory response. This hypothesis should be evaluated in future experiments where the levels and exposure time patterns of endotoxin and $\beta$(1,3)-D-glucan including other forms of glucan than the one studied here, should also be investigated. In such experiments, functional studies of macrophages and other inflammatory cell types could yield important additional information on the agents postulated to be of importance for the development of HP.

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