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Experimental allergic alveolitis after exposure to different microorganisms

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Summary. The experiments described here examined the capacity of different microorganisms to induce allergic alveolitis. Guinea-pigs were exposed to an aerosol of pure cultures of five different organisms, four of which are common in mouldy hay, without previous injection of an adjuvant. The animals were either acutely exposed or exposed for 3 to 5 weeks, after which the numbers of different inflammatory cells in the airways were counted and histological changes in the lung parenchyma were assessed. It was seen that prolonged exposures to large numbers of spores produced a cellular infiltration in the alveolar and bronchiolar region, and gave rise to lesions resembling early granulomas. The number of lymphocytes increased in the airways. The results suggest that allergic alveolitis can be induced by inhalation of various kinds of microorganisms and that these may vary in their capacity to produce the disease.

Keywords: allergic alveolitis, mould, granuloma, free lung cells

The risk for developing allergic alveolitis (AA) has been associated with a variety of environments that involve exposure to organic dusts (Rylander & Petersson 1990). Common to many of these environments is the presence of microbial aerosols, particularly moulds. It has been suggested that the risk of AA among farmers is considerably increased when exposure exceeds roughly 10^8 spores/m^3 (Larsson *et al.* 1988).

Among the different kinds of microorganisms present in various environments, *Micropolyspora faeni* has historically attracted most interest in connection with exposure to mouldy hay. However, it is now realized that AA may also develop after exposure to other microorganisms, such as *Streptomyces* and *Aspergillus* (Terho & Lacey 1979), although different species of microorganisms may have different capacities to induce AA (Lacey 1975). Knowledge about the relative risk for AA after exposure to different organisms is useful for risk evaluations and preventive purposes, particularly when new environments are to be evaluated.

Apart from microorganisms, bacterial endotoxins are often present in environments associated with AA. Previous studies have shown that the cellular reactions induced by inhaled pure endotoxin differ from those caused by mouldy hay (Fogelmark *et al.*, 1989). Nevertheless, endotoxins

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could play a role in the initial inflammatory process leading eventually to AA.

A risk evaluation of effects after chronic exposure to microorganisms requires animal models. Several such models have described AA, most involving the injection of an adjuvant, followed by an intratracheal injection or, in a few cases, inhalation of the suspected antigen (e.g. Schuyler *et al.*, 1987, Richerson *et al.*, 1982). We recently described an animal model involving inhalation of an aerosol without previous injection of an adjuvant (Fogelmark *et al.*, 1989). The chief features of this model are a sufficiently high concentration of airborne spores, a daily exposure of 4 h and a duration of 5 weeks.

In the present experiments, we studied the capacity of different microorganisms to induce AA. Animals were exposed to an aerosol of pure cultures of five different organisms, four of which are commonly found in mouldy hay and other environments where AA has been described.

Guinea-pigs were either acutely exposed or exposed for 3 or 5 weeks, after which the numbers of different inflammatory cells in the airways were counted and the histological changes in the lung parenchyma were assessed.

Materials and methods

Animals

Guinea-pigs of both sexes weighing 300–500 g were placed in exposure chambers for 4 h daily and then kept in animal cages supplied with filtered air at a slight positive pressure. Food and water were supplied *ad libitum*.

Microorganisms

Spores of *Aspergillus fumigatus*, *Faenia rectivirgula* (Micropolyspora faeni), *Phanerochaete chrysosporium*, *Rhizopus stolonifera* and *Penicillium aurantiogriseum* were produced by culturing them on rice. Parboiled rice was rinsed under lukewarm water until the rinse water was clear. Moist rice (60 g) was weighed into 1-l flasks and autoclaved at 120°C for 20 min. The flasks were inoculated with 3 ml of a spore suspension of the organism studied (10^7/ml 0.9% NaCl) and incubated at38°C for the *Aspergillus*, *Phanerochaete*, *Rhizopus* and *Penicillium*, and at 50°C for *Faenia*. After an incubation time of 3–6 days, the rice culture was air dried.

Exposure

The dried rice colonized by microorganisms was placed in a horizontal drum (length 70 cm, diameter 40 cm) rotating at 10 r.p.m. to generate an aerosol of the spores which was then passed into an exposure chamber. The concentration of the aerosol was changed by using different quanties of rice in the drum and by altering the proportions of fresh and aerosol-laden air entering the exposure chamber.

The aerosol in the exposure chamber was monitored by drawing 2 l air/min through a Millipore filter (HAWP pore size 0.8 μm). After exposure, the filters were removed and spores suspended by shaking the filter in 10 ml saline with one drop of 2% saponine. The suspensions were then examined under a microscope.

Ninety-eight per cent of the particles collected on the filter consisted of spores from the microorganism studied. The number of spores was counted in a Bürker chamber and the concentration of spores/m^3 was calculated. In view of the variation in numbers, the closest power of 10 of the mean value was taken as the actual dose.

The target exposure level was 10^9 spores/m^3. However, preliminary experiments demonstrated that *Aspergillus* was very toxic to the animals at that dose and few survived even single exposures. The exposure dose for the various organisms studied was thus titrated using experience from the acute exposure and the final concentrations ranged from 10^6 to 10^9 spores/m^3. The amount of airborne endotoxin was deter-

Table 1. Exposure conditions

Exp.	Organisms	Spores/m	Endotoxin ($\mu g/m^3$)
1	*Aspergillus fumigatus*	7×10^5	0.0001
2	*Aspergillus fumigatus*	3×10^7	0·002
3	*Faenia rectivirgula*	3×10^8	0.13
4	*Rhizopus stolonifera*	2×10^7	0.04
5	*Phanerochaete chrysosporium*	2×10^9	0.14
6	*Penicillium aurantiogriseum*	1×10^9	0.003

mined in all experiments using the chromogenic version of the Limulus lysate assay, as previously described (Rylander & Morey 1982; Goto & Rylander 1987). The exposure conditions in the different experiments are summarized in Table 1.

Animals were examined after an acute exposure (4 h) and at 3 and 5 weeks (4 h/day, 5 days/week) after the start of the exposure. A 24-h period was always allowed between the last exposure and killing the animals. For each experiment, control animals from the same batch of guinea-pigs were maintained in cages supplied with filtered air during the experiment period. In some studies, control animals were examined at the beginning and end of the experiment.

Examination

After a lethal i.p. dose of pentobarbital, the main bronchus of the left lung was ligated. The right lung was subject to lung lavage using 5 ml of saline which was slowly injected into the lung and withdrawn. This procedure was repeated 10 times with the same fluid. A sample of the fluid was stained with Türk's solution and the number of cells counted. A smear of the cells was prepared using a cytocentrifuge, stained with May–Grünwald–Giemsa stain and the numbers of different cell types were determined.

The left lung was removed and fixed by slowly injecting 4% buffered formaldehyde. 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 location; 5, defined granulomas. The severity index for the histological changes in a group of animals was calculated as the mean of each animal score.

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. The Mann–Whitney *U*-test was used for histological preparations.

As the results from the control animals were very similar in the experiments, these groups were pooled in the statistical evaluation. In view of the high number of degrees of freedom, $P < 0.001$ was chosen as the level of significance.

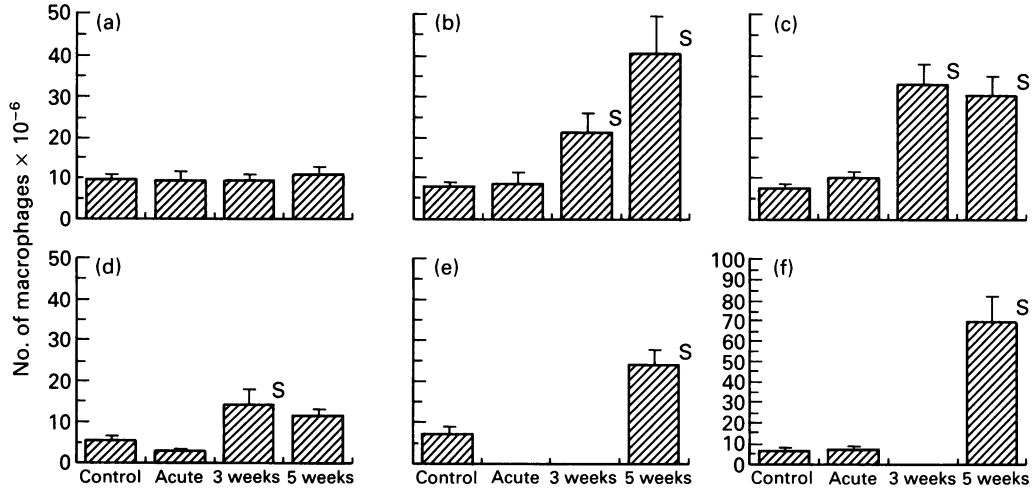

Fig. 1. Number of macrophages ($\times 10^{-6}$) in airways of animals exposed to aerosols of different microorganisms acutely or for 3 or 5 weeks. Bars indicate s.e. a, *Aspergillus* ($10^6/m^3$); b, *Aspergillus* ($10^7/m^3$); c, *Faenia* ($10^8/m^3$); d, *Rhizopus* ($10^7/m^3$); e, *Phanerochaete* ($10^9/m^3$); f, *Penicillium* ($10^9/m^3$). S, Statistical significance, $P<0.001$.

Results

The animals tolerated the exposure well, and there were no outward signs of respiratory impairment or generalized disease at the end of the exposures.

Figure 1 illustrates the number of macrophages in the lung lavage of animals exposed to the different organisms. In this and the following figure control values are those from the particular experiment. Exposure to *Aspergillus* at a concentration of $10^6/m^3$ did not affect the number of macrophages. At $10^7/m^3$, the number of macrophages was significantly higher at 3 and 5 weeks. Similarly, exposure to *Faenia* at $10^8/m^3$, *Phanerochaete* at $10^9/m^3$ and *Penicillium* at $10^9/m^3$ caused a significant increase in the number of macrophages after 3 and 5 weeks' exposure. The number was also increased after exposure to *Rhizopus* at $10^7/m^3$ after 3 weeks of exposure, but after 5 weeks was not significant ($P<0.003$).

Figure 2 demonstrates the number of lymphocytes in the lung lavage. The reaction pattern was similar to that observed for macrophages except that there was also a significant increase at 3 and 5 weeks in animals exposed to *Aspergillus* at $10^6/m^3$ and at 5 weeks after exposure to *Rhizopus*. However, the relative increase in the number of lymphocytes was largest after the exposure to *Faenia* and *Penicillium*. The same pattern was seen for the number of neutrophil and eosinophil leucocytes (Figs 3 and 4). In addition, a significantly larger number of the latter cells was also found after the acute exposure to *Faenia* as compared to unexposed animals.

The histological changes in the lungs of exposed animals are illustrated in Fig. 5. The irritation index was larger in animals exposed for 3 and 5 weeks than in control animals for all organisms. These changes were both interstitial and intra-alveolar and increased with an increasing exposure time. They were seen predominantly around bronchioles and not around blood vessels. This cellular response increased with exposure time up to 5 weeks in these studies. No

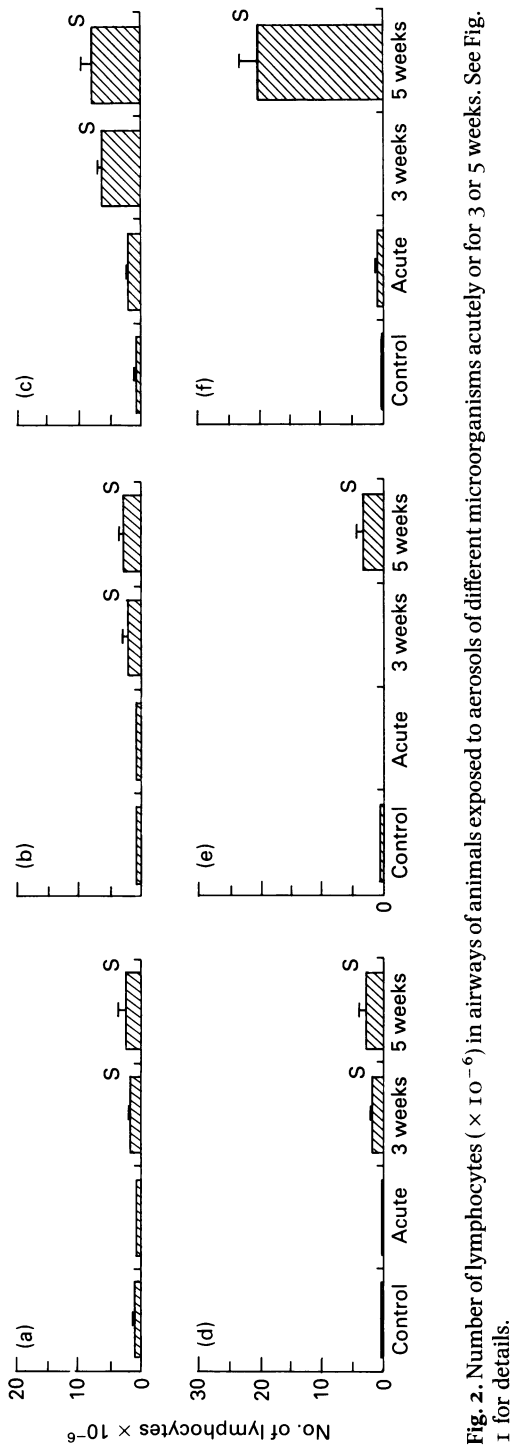

Fig. 2. Number of lymphocytes ($\times 10^{-6}$) in airways of animals exposed to aerosols of different microorganisms acutely or for 3 or 5 weeks. See Fig. 1 for details.

apparent fibrosis was found. The changes were most pronounced after exposure to *Aspergillus* at $10^7/m^3$.

Discussion

The diagnosis of allergic alveolitis is based on history of exposure to typical environments or symptoms and clinical findings, as well as histopathology (Terho 1982). The latter comprises an increased number of lymphocytes in bronchoalveolar lavage fluid, monocyte infiltration and granulomas in the lung parenchyma (Richerson 1983).

The results from the present experiments confirm our previous observations using mouldy hay as an exposure agent. A prolonged exposure at high spore levels produced a cellular infiltration in the alveolar and bronchiolar region, with some lesions resembling early granulomas and increases in the number of lymphocytes in the airways studied by lung lavage. This histopathology resembles findings in the early stages of AA, in which an increased number of lymphocytes has been found among persons exposed to organic dusts (LeBlanc *et al.*, 1986; Reynolds *et al.*, 1977). In contrast to many reports of clinical cases of AA, an increased number of neutrophils was found in lung lavage fluid. This is probably because the animals were sampled relatively shortly after the exposure, whereas clinical cases are often examined later. This explanation is consistent with findings from persons with AA who were challenged with the appropriate antigen (Fournier *et al.*, 1985), in whom the number of neutrophils was elevated in lung lavage. An increased number of neutrophils has also been found in lungs of farmers who suffer from toxic alveolitis (organic dust toxic syndrome) and who also demonstrated an increase in the number of lymphocytes in the airways, indicative of early AA (May *et al.*, 1986).

Classic AA comprises extensive cell infiltrates, granulomas and fibrosis, a clinical picture of severe pulmonary disease (Terho 1982). However, it is now realized that, in a

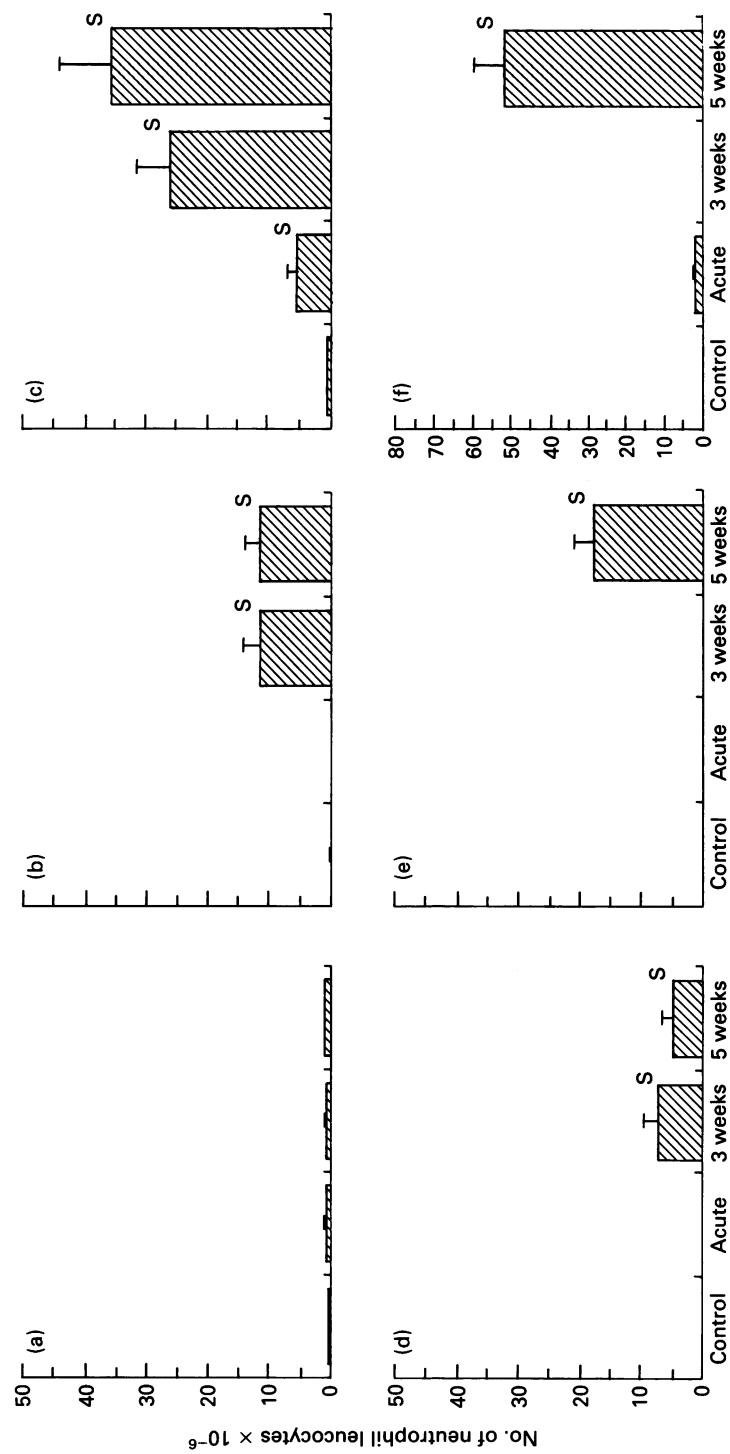

Fig. 3. Number of neutrophil leucocytes ($\times 10^{-6}$) in airways of animals exposed to aerosols of different microorganisms acutely or for 3 or 5 weeks. See Fig. 1 for details.

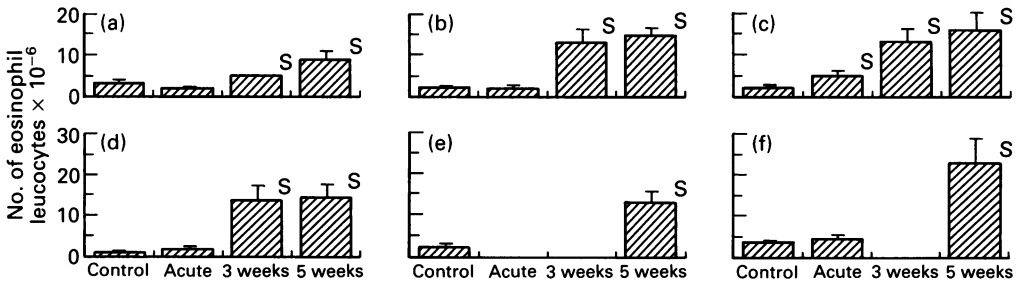

Fig. 4. Number of eosinophil leucocytes ($\times 10^{-6}$) in airways of animals exposed to aerosols of different microorganisms acutely or for 3 or 5 weeks. See Fig. 1 for details.

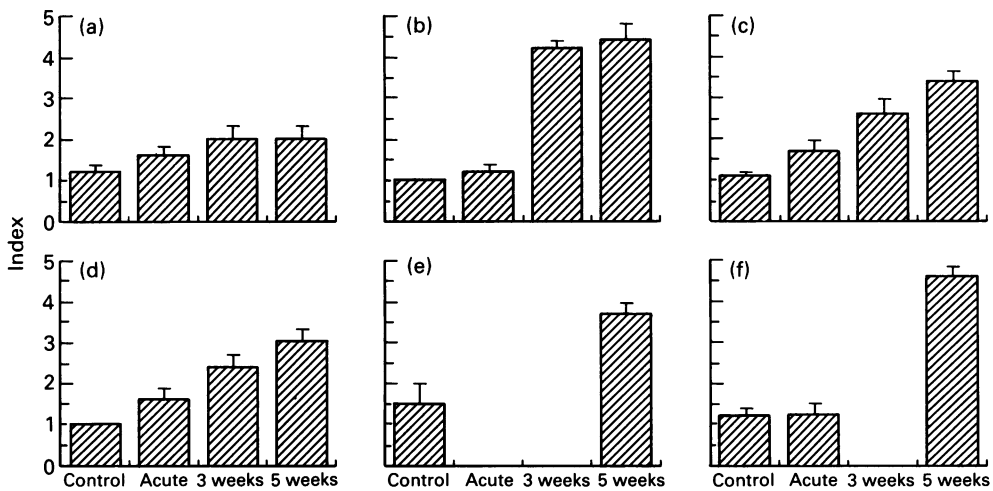

Fig. 5. Histological changes (index see text) in airways of animals exposed to aerosols of different microorganisms acutely or for 3 or 5 weeks. See Fig. 1 for details.

population of persons exposed to organic dusts, for example farmers, there are several different stages of AA. An increase in the number of lymphocytes in the airways as well as slight inflammation, involving an invasion of lymphocytes and neutrophils into the airways, are relatively common and have been described in 1% or more of farmers, as compared to the much lower incidence of 2–3 per 10 000 for AA with fibrosis (Malmberg *et al.*, 1988). It is conceivable that the various stages of AA represent different phases of activation of the inflammatory system and that special mechanisms are involved when fibrosis develops. The histopathological findings in our experiment thus represent an early stage of AA.

Pulmonary fibrosis was not an endpoint in the present animal model. As discussed above, this is a relatively rare phenomenon in a population of workers exposed to organic dusts and, until further insight is gained into the mechanism behind the development of fibrosis in a few of the exposed persons, an

animal model with this endpoint cannot be developed.

The results demonstrate that different organisms have different potentials for causing AA. *Aspergillus* at a level of $10^7/m^3$ gave rise to nearly the same changes in lung lavage cells as *Faenia* at $10^8/m^3$. Exposure to *Penicillium* at $10^7/m^3$ brought about no change in the number of free lung cells (data not shown), whereas an exposure to *Rhizopus* and *Aspergillus* at the same level caused an increase in the number of lung lavage cells.

The results also demonstrate that all organisms tested induced histological and inflammatory cell changes resembling AA. The development of AA is thus not confined to one or a few organisms but could probably be expected in any environment in which the amount of airborne microorganisms is sufficiently high.

If the capacity to induce a chronic granulomatous inflammation in the lung is a general phenomenon for many organisms, a causative agent must be a general component of the organisms and have a defined biological activity. Several candidates can be suggested.

Cytochalasins are a group of fungal metabolites which demonstrate specific biological effects such as inhibition of cell movement, cell division and phagocytosis. This prevents cell communication and interferes with the inflammatory and immunological responses to inhaled agents (Thilly *et al.* 1978).

Lectins are a group of compounds defined by their ability to bind carbohydrates and can be isolated from a wide variety of sources such as microorganisms and plants. Their function in nature has not yet been explored, but the binding properties and specificity of some lectins resemble that of antigen to antibody, and their biological activity, for example their mitogenicity, is considerable (Bøg-Hansen & Freed 1988).

Another group of substances potentially involved is the glucans, among which β-1,3-glucans seem to be of special interest. They are potent immunomodulators (Di Luzio 1985), have antitumour activity (DiLuzio 1979) and stimulate the RES (Cook *et al.*, 1980). They appear as structural components and metabolic products of microorganisms.

For future work on causative agents of AA, it will be necessary to assay each of the above components separately with regard to their potential for inducing a granulomatous inflammation in the lung.

Apart from the spore particles, the aerosols in the present experiment also contained endotoxin, probably because of contamination during the culture. This contamination could not be avoided with the culturing technique used here, as rice is naturally contaminated with Gram-negative bacteria (Olenchock *et al.*, 1984) and autoclaving does not destroy the biological activity of their endotoxin.

Endotoxins are well known mitogens and may act as adjuvants, thus potentiating the effect of moulds, and increasing the severity of the inflammation. Bacteria and bacterial endotoxin are also common components of organic materials. As demonstrated previously, the inflammatory response after exposure to endotoxin is rather different from the one induced by mouldy materials (Fogelmark *et al.*, 1989). In addition, the most pronounced reactions in this experiment were caused by the organisms with the lowest contamination of endotoxin (Table 1). However, their possible contribution to the development of AA should be further investigated.

In conclusion, the results from the present study suggest that AA can be produced by inhalation of isolated microorganisms of various kinds and that they may vary with regard to their potential for inducing AA. The present animal model can be used to assess the importance of various subcomponents of the microorganisms for the development of AA.

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