



The fungal biopesticide *Metarhizium anisopliae* has an adjuvant effect on the allergic response to ovalbumin in mice

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Received 27 April 2005; received in revised form 8 September 2005; accepted 9 September 2005

Abstract

The parasitic fungus, *Metarhizium anisopliae*, is non-pathogenic to humans and licensed for indoor control of cockroach infestation. An important reason for the elimination of this vermin is that sensitisation to cockroaches is associated with asthma. Previously *M. anisopliae* has been shown to cause allergic- and asthma-like responses in mice and in the present study we have examined the adjuvant activity of *M. anisopliae* on the allergic response to the model allergen ovalbumin (OVA) in a mouse model. Levels of OVA-specific IgE, IgG1 and IgG2a in serum were measured and the weight and cell number of the excised popliteal lymph node were determined. Mice primed with mycelium + OVA and boosted with OVA had increased anti-OVA IgE and IgG1 levels compared with mice primed with OVA alone or mycelium. Priming with *M. anisopliae* (as mycelium or MACA) increased weight or cell number of the excised PLNs. These results suggest that *M. anisopliae* has the ability to increase an allergic response to an allergen and consequently, may worsen allergy in susceptible individuals.

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Keywords: Adjuvant effect; IgE; *Metarhizium anisopliae*; Mice; Mould

1. Introduction

Allergy and asthma are common disorders causing severe health problems. Several factors in the indoor environment including allergens, endotoxin and mould have been associated with such illnesses (Kurup et al., 2000; Liu, 2004). For over 30 years cockroach allergy has been recognised as an important cause of asthma (Arruda et al., 2001; Bernton et al., 1972) and exposure and sensitisation to cockroach allergen is associated

with increased asthma morbidity in children living in inner cities in the US (Arruda et al., 2001). Furthermore, cockroach infestations of houses are an important public health problem. The prevalence of cockroach sensitisation has been recorded to be from 17% to 41% in the United States (Arruda et al., 2001), from 11% to 47% in Asia (Bener et al., 2002; Kim et al., 2001) and from 4% to 26% in Europe (Hirsch et al., 2000; Sastre et al., 1996) depending on the group studied.

Metarhizium anisopliae is an entomopathogenic fungus licensed for indoor control of cockroaches. The conidia are the viable reproductive unit of the fungus and the “active ingredient” for commercial application of *M. anisopliae* (Roberts and St Leger, 2004). The conidia attach to the surface of the cockroach and penetrate

Abbreviations: MACA, *Metarhizium anisopliae* crude antigen; OVA, ovalbumin; PLN, popliteal lymph node

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the cuticle by appressoria (i.e. penetrating structures) (Vestergaard et al., 1999). In addition, proteases, chitinases (Krieger et al., 2003; St Leger et al., 1998) and the toxic destruxins are released (Pedras et al., 2002). A correlation between production of destruxins and fungal virulence has been suggested (Pedras et al., 2002), but the effectiveness of the fungus as a pesticide depends on its ability to spread throughout the cockroach colony.

When using the pesticide indoor, inhalable particles of fungal spores, mycelium and enzymes, in combination with cockroach allergen, will most likely be present in the indoor environment. The pesticide has been found to be non-pathogenic for humans, but until recently little research had focused on its potential to induce allergy. There is limited clinical data suggesting that some individuals exposed occupationally to *M. anisopliae*, have become sensitised (Kaufman and Bellas, 1996). In studies, Ward et al. (Ward et al., 1998, 2000b) have shown that BALB/c mice exposed to MACA develop allergy/asthma-like responses. Additionally, they have detected potent allergens of *M. anisopliae* (Copeland et al., 2002).

Previously, we have found that mould extracts of *Cladosporium herbarum* and *Penicillium chrysogenum*, as well as the mould cell wall component β -1,3-glucan, increase the allergic response to a model allergen, ovalbumin (OVA) in mice (Ormstad et al., 2000; Instanes et al., 2004). Moulds and their cell wall component β -1,3-glucan have demonstrated adjuvant effects. Additionally, the fungal biopesticide *M. anisopliae* has demonstrated allergic potential. The question is whether or not this allergenic mould can exacerbate the allergenicity of the pest it is used to control. If so, then an already susceptible human population may be exposed to increased risk. The aim of the present study was to evaluate the potential for the fungal biopesticide *M. anisopliae* to generate adjuvant effects on the induction of allergy against a model allergen.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6–8 weeks old) were obtained from Gl. Bomholtgård, Ry, Denmark. They were rested for 1 week after arrival, housed eight per cage and were individually marked by ear punching. The mice were given water and food ad libitum. The experiments were performed in conformity with the laws and regulations of animal experiments in Norway, and approved by the local officer of the Animal Board under the minister of Agriculture in Norway.

2.2. Fungal antigen preparation

The fungal antigen preparation was performed as previously described (Ward et al., 1998). In short, *M. anisopliae* strain 1080 was obtained from USDA-ARS Entomopathogenic Fungus Collection in Ithaca, NY. *M. anisopliae* mycelium and conidia proteins were extracted in Hank's balanced salt solution (HBSS; Gibco BRL, Grand Island, NY). Subsequently, the protein extracts were concentrated using Amicon stirred cell concentrator with an YM3 filter (MWCO 3000) (Amicon, Beverly, MA). Additionally, a filtrate of proteases and chitinases (inducibles) induced in a deprived media (chitin and water) were concentrated as described above. The extract concentrations were determined using Pierce Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) and a BSA (Sigma Chemical Co, St. Louis, MO, USA) to produce a standard curve. The three extracts (mycelium, conidia and inducible enzymes) were all diluted to 0.5 mg/ml in phosphate-buffered saline (PBS) medium (PBS with 10% BALB/c mouse serum) and combined in equal protein amounts to form *M. anisopliae* crude antigen (MACA). MACA was found to be endotoxin free at 0.125 mg/ml in pyrogen-free saline by Limulus amoebocyte lysate analysis (Pyrotell®, Associated of Cape Cod Inc., East Falmouth MA).

2.3. Preparations for immunisation

The priming doses were 30 μ g of MACA alone (10 μ g mycelium + 10 μ g conidia + 10 μ g inducible enzymes), 30 μ g of MACA together with 10 μ g of OVA (cat. no. A7641, Sigma Chemical Co.), 25 μ g of mycelium alone or 25 μ g of mycelium together with 10 μ g of OVA. The injected concentrations, 30 μ g and 25 μ g of MACA and mycelium, respectively, were given in 20 μ l, while the injected concentration of OVA was 10 μ g /20 μ l. All solutions were dissolved in PBS medium.

2.4. Experimental design

Mice were bled from the distal part of the saphenous vein at day –1 (before the start of the experiment). On day 0, groups of eight mice were injected subcutaneously (s.c.) with 20 μ l of one of the following preparations: MACA + OVA, MACA alone, mycelium + OVA, mycelium alone, OVA alone, PBS medium or untreated. Solutions were injected into the right hind footpad (heel-toe direction) using a 100 μ l Hamilton syringe (Hamilton, Bonaduz AG, Switzerland) with a 30-G needle (Becton, Dickinson & Co, Dublin). On day 21 all the mice were injected with OVA alone into the same footpad, except for untreated mice and mice given PBS medium alone. On day 26 all the mice were anaesthetised with CO₂ and terminated by heart puncture (Fig. 1). The PLN from the right hind leg was removed and stored in HBSS. It was stripped of fat and weighed. Each lymph node was then placed in 200 μ l of HBSS on a petri dish and teased into a single cell suspension using 18-G needles. Cell suspensions were enumerated using a cell counter (Coulter

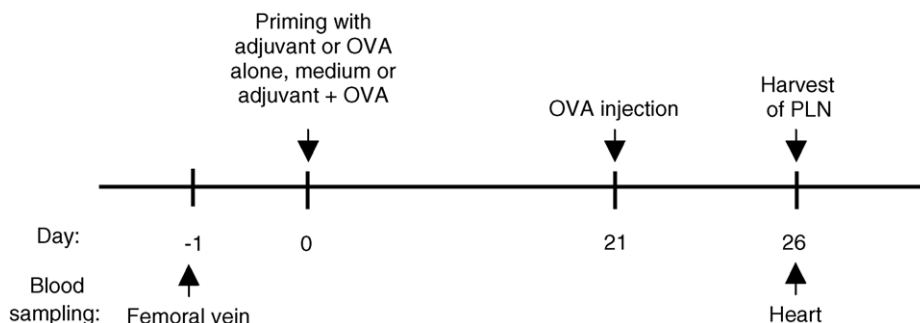


Fig. 1. Sensitisation and sampling protocol.

Counter Z1, Beckman Coulter Incorporated, FL, USA). In one experiment with MACA the PLN were frozen prior to handling. To measure the local cellular response, weight and cell number of the right lymph node (inoculated) was used. Previously, indices for weight and cell number of the right PLN divided by that of the left PLN (untreated) have been used to measure the local cellular response (Bloksma et al., 1995). The weight of lymph nodes from untreated animals varied less than expected. Because preliminary studies show that the left lymph node is affected by the treatment of the right hind footpad, we used only the lymph nodes from the treated side to compare differences in treatment.

2.5. Collection of blood samples

Blood samples were drawn from the distal part of the saphenous vein (Aaberge et al., 1992). After removing hair from the area with a scalpel blade, the vein was punctured with a 21-G needle and 200 μ l of blood collected into heparinised capillary tubes (Vitrex, Herlev, Denmark). Serum from all blood samples was prepared and stored at -20°C until analysed.

2.6. Assay for measurement of IgE and IgG1 anti-OVA by ELISA

The procedures have been described in detail elsewhere (Lovik et al., 1997; Ormstad et al., 2000). In short, to detect IgE anti-OVA, microtitre plates were coated with rat anti-mouse IgE antibody (clone LOME-3, Serotec, Oxford UK). Then the plates were added sera and incubated subsequently with biotinylated OVA, streptavidin in complex with biotinylated alkaline phosphatase (ABComplex/AP, DAKO, Glostrup, Denmark), and at least *p*-nitrophenyl phosphat disodium hexahydrat diluted in diethanolamin-buffer. Standard curve of mouse serum pool containing defined amounts of IgE anti-OVA, were included on each plate. Optimal density (OD) was measured at 405 nm. Measurement of IgG1 was done in a similar way as described above, but the plates were coated with rat anti-mouse IgG1 (clone LO-MG1-13, Serotec Ltd., Oxford, UK). Lower detection limits for IgE and IgG1 were 0.5 and 0.02 AU/ml, respectively.

2.7. Assay for measurement of IgG2a anti-OVA by ELISA

The procedures have been described in detail elsewhere (Nygaard et al., 2005). In short, to detect IgG2a anti-OVA, microtiterplates were coated with OVA and blocked with Tris/Tw with 1% BSA. Then the plates were added sera and incubated with anti-mouse IgG2a (cat. no. 02012D-553388, BD Biosciences PharMigan, San Diego, California, USA). ELISA was developed using IgG2a binding streptavidin in complex with biotinylated alkaline phosphatase (ABComplex/AP, DAKO), and at least *p*-nitrophenyl phosphat disodium hexahydrat diluted in diethanolamin-buffer. Standard curve of mouse serum pool containing defined amounts of IgG2a anti-OVA, were included on each plate. Optimal density (OD) was measured at 405 nm. Lower detection limits for IgG2a was 0.02 AU/ml.

2.8. Statistical analysis

Data were analysed with SigmaStat[®] Statistical Analysis System for the PC Version 2.03 (Jandel Scientific, Erkrath, Germany). Equivalent to one-way analysis of variance, the Krustal-Wallis one-way analysis on ranks (non-parametrical test) was used to compare all groups. Then a multiple comparison versus one control group (Dunnet's method) was performed. The limit for statistical significance was set at $p = 0.050$.

3. Results

3.1. Effects of mycelium and MACA on the systemic anti-OVA response

Mice primed with mycelium + OVA had enhanced serum levels of anti-OVA IgE and IgG1 (more than 4-fold ($p < 0.05$)) compared to those primed with OVA alone (Fig. 2a and b). Moreover, the groups primed with mycelium alone or mycelium + OVA had increased levels of OVA-specific IgG2a (more than 2-fold ($p < 0.05$)) compared to mice primed with OVA alone (Fig. 2c). However, priming with MACA alone or MACA + did not

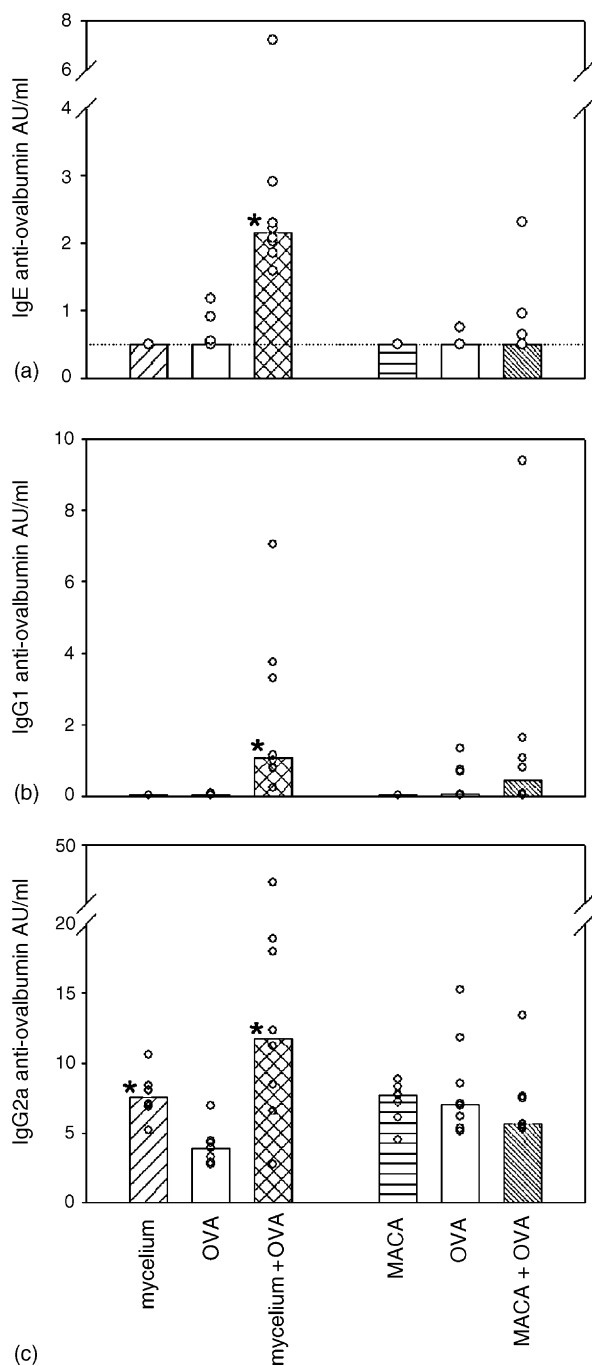


Fig. 2. Levels of OVA-specific IgE (a), IgG1 (b) and IgG2a (c) were measured in mouse serum on day 26. On day 0, mice were primed with mycelium (25 μ g) ($\square$), MACA (30 μ g) ($\square$), or OVA alone (10 μ g) ($\square$) or either mycelium + OVA ($\boxtimes$), MACA + OVA ($\boxtimes$). At day 21 all mice were injected with 10 μ g of OVA. Values are given in arbitrary units (AU) for each individual mouse (circle) and median for groups of eight mice (columns). Dotted line shows the lower detection limit for IgE (0.5 AU/ml) (* $p < 0.05$ compared with OVA alone).

significantly affect the levels of OVA-specific IgE, IgG1, and IgG2a (Fig. 2). Mice untreated or primed with PBS medium did not show any antibody response to OVA. In a second experiment the immunoglobulin pattern was confirmed (data not shown).

3.2. Effects of mycelium and MACA on the local PLN response

On day 26, five days after all mice received an injection of OVA, the weight and cell number of the PLN increased approximately 2-fold ($p < 0.05$) in the group primed with mycelium + OVA compared with the group primed with OVA alone (Fig. 3). In mice primed with mycelium alone, only the weight of PLN was increased (2-fold) compared with those primed with OVA only (Fig. 3). In mice primed with MACA + OVA (Fig. 4), the cell number, but not the weight, of the PLN was significantly higher (2-fold) compared with the group primed with OVA ($p < 0.05$) (Fig. 4). However, there was no adjuvant effect when priming the animals with MACA alone (Fig. 4). The generally lower number of cells in the experiment is due to freezing of these particular PLN before handling. The weight and cell number of PLN in mice primed with PBS medium did not increase. These

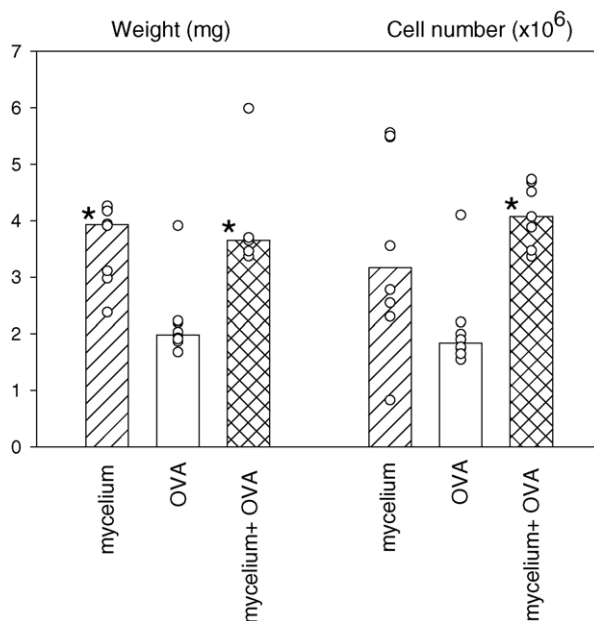


Fig. 3. Weight (mg) and cell number ($\times 10^6$) of the right hind leg of mice were measured on day 26 after priming with mycelium (25 μ g) ($\square$), mycelium (25 μ g) + OVA (10 μ g) ($\boxtimes$) or OVA (10 μ g) ($\square$) and injection of OVA (10 μ g) on day 21. Values are shown for individual mouse (circle) and median for groups of eight mice (columns) (* $p < 0.05$ compared with OVA alone).

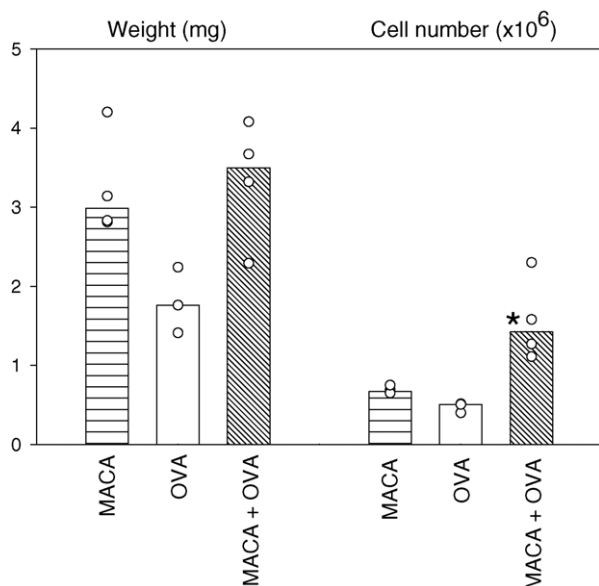


Fig. 4. Weight (mg) and cell number ($\times 10^6$) of PLN (y-axis) of the right hind leg of mice on day 26 after priming with MACA (30 μ g) ($\equiv$), MACA (30 μ g) + OVA (10 μ g) ($\blacksquare$) or OVA alone (10 μ g) and injection of OVA (10 μ g) ($\square$) on day 21, were measured after freezing. Values are shown for individual mouse (circle) and median for groups of four mice (columns) (* $p < 0.05$ compared with OVA alone).

findings were confirmed in a second experiment (data not shown).

4. Discussion

M. anisopliae, licensed as an indoor pesticide to control cockroach infestation, has been shown to cause allergy and asthma like-responses in mice (Ward et al., 1998, 2000a,b). In the present study we investigated whether or not *M. anisopliae*, like other moulds and the cell wall component β -1,3-glucan (Ormstad et al., 2000; Instanes et al., 2004), has adjuvant effects in a mouse model for allergy. We report that there are components of *M. anisopliae* that increase the allergic response to OVA in this model.

The footpad injection model used in our experiments, is well established for screening of chemical allergens and immunotoxic substances (Bloksma et al., 1995; Descotes and Verdier, 1995; Pieters and Albers, 1999). Additionally, it is useful for the identification of adjuvants (Lovik et al., 1997). However, it is important to administer *M. anisopliae* by the airways, the most relevant exposure route, in order to draw firm conclusions.

In this study we chose to examine the mycelium of *M. anisopliae* because in preliminary studies (Copeland et al., 2002) more IgE-inducing proteins were detected in this fungal component than in the conidia. The fun-

gal extracts used in this study, have also been used in other studies (Ward et al., 1998, 2000a,b). A total MACA concentration of 30 μ g in 20 μ l, containing 10 μ g of mycelium, did not reveal an increased antibody response. However, when a higher concentration (25 μ g in 20 μ l) of the mycelium fraction was used, there was such a response. The lack of an enhanced OVA response may be due to lower mycelium concentration or inhibitory factors in the other fungal extracts that make up MACA.

Extracts of *M. anisopliae* were either given alone or together with OVA in mice that were boosted with OVA 20 days later, to study a primary or secondary response to OVA, respectively (Fig. 1). We examined the local and systemic response to OVA. The local response was measured in the PLN in mice given *M. anisopliae*, either as MACA or mycelium. Both the primary and secondary response to OVA tended to increase the weight and cell number of the PLN. This result is in accordance with previous studies in which exposure to MACA have caused an increased number of cells in the lungs of mice (Ward et al., 1998, 2000a,b).

The systemic response was examined by measuring levels of the commonly known Th2-associated IgE and IgG1 antibodies and Th1-associated IgG2a antibodies. The levels of OVA-specific IgE and IgG1 increased significantly in the secondary response compared with the primary response to OVA in mice given mycelium of *M. anisopliae*. Moreover, the response was also increased compared with mice given OVA alone twice. This indicates that the mycelium of *M. anisopliae* has the ability to increase the allergic response to an allergen (OVA) when the allergen is boosted. Even though mice primed with MACA + OVA had seven times higher IgG1 anti-OVA levels than mice primed with OVA alone (Fig. 2b), the increase was not statistically significant, most likely due to a large variation. Moreover, neither the IgE nor the IgG2a anti-OVA responses were increased significantly. The dose given of isolated mycelium was 2.5 times higher than the dose of mycelium contained within MACA. This difference may explain the lack of a significant response with MACA in the current model, which implies a dose-dependent effect of mycelium. Unfortunately, due to upper limit of 20 μ l as the injected volume, we were unable to test a higher concentration of MACA in this model.

Most interestingly, the secondary response to OVA in serum to mice given *M. anisopliae* mycelium together with OVA, not only increased the levels of OVA-specific Th2-associated IgE and IgG1 antibodies, but also the specific Th1-associated IgG2a antibody response was increased compared with mice given OVA. Also, in the primary response to OVA in mice primed with mycelium,

the IgG2a response was significantly increased. This indicates that *M. anisopliae* is able to raise both Th1- and Th2-associated responses in mice. The Th2-associated response was only observed as a result of a secondary antibody response, while the Th1-associated response was initiated both as a result of primary and secondary response to OVA. Since the primary response was strong even when OVA was given 20 days after mycelium and only five days before the termination, priming with *M. anisopliae* must induce a well suited Th1-associated environment in the mice. An increase in antigen-specific IgE, IgG1 and IgG2a antibodies in mice exposed to adjuvant and allergen was also found in similar studies (Bunder et al., 2004; Ormstad et al., 2003). Furthermore, Th1 cells have been found to cooperate with Th2 cells in airway inflammation rather than counterbalancing the Th2-cell response (Hansen et al., 1999; Randolph et al., 1999). This indicates that the Th1/Th2 paradigm, in which Th1 cells down-regulate allergic diseases, may be more complex than initially suggested. Moreover, there is no valid evidence for a protecting or increasing role of IgG in the allergic response (Poulsen, 2000). However, an increase of IgE antibodies is most strongly related to allergy.

β -Glucans are commonly found in the cell wall of fungi, some plants and bacteria (Young et al., 1998). These glucose polymers are known immunomodulators (Riggi and Di Iuzio, 1961). β -1,3-Glucan increase the allergic response in mice, but only in relatively high doses (Ormstad et al., 2000; Instanes et al., 2004). In a similar study we previously suggest that the adjuvant effect of extracts of *Cladosporium herbarum* and *Penicillium chrysogenum* was not caused by β -glucan alone at low dose, but more likely by other adjuvants (Instanes et al., 2004). Both the β -glucan content and structure of *M. anisopliae* is unknown. However, compared with the doses of β -glucan that had effect in the previous study (100 μ g), the effect found in the present study is most likely caused by other adjuvants in addition to β -glucan.

In accordance with previous experiments with the common indoor moulds *Cladosporium herbarum* and *Penicillium chrysogenum* (Instanes et al., 2004), *M. anisopliae* also has the ability to increase an allergic response to an allergen. Although further studies of moulds are warranted for a conclusion, these findings indicate that moulds in general contain potent compounds that enhance the development of allergy. Furthermore, elimination of cockroaches is a prerequisite for removal of the potent cockroach allergens in the indoor environment, which may induce and trigger asthma. However, previous studies indicate that the fungal biopesticide *M. anisopliae* has the potential to

induce allergy/asthma due to self-contained allergens (Ward et al., 1998, 2000a,b). Additionally, the present study suggests that *M. anisopliae* also produce one or more adjuvants. Hence, in susceptible individuals, this fungus may increase the allergic response to its own fungal allergens as well as to other indoor allergens, such as cockroach allergens. Although *M. anisopliae* is considered to be of low toxicity to humans, previous and present studies imply that it is questionable to use this fungus as a biopesticide in homes of individuals with allergy or asthma.

Acknowledgements

We want to thank Else-Carin Groeng and Åse Eikset for excellent technical support and The Norwegian Research Council for financial support of this work. This research paper has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflects the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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