

Mould extracts increase the allergic response to ovalbumin in mice

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Summary

Background Exposure to moulds in indoor air is thought to induce asthma in susceptible persons. Moulds may contain several potent allergens. However, more importantly, moulds may increase the allergic response to other allergens (adjuvant effect). Previously, we have found that a β -1,3-glucan from the cell wall of the fungus *Sclerotinia sclerotiorum* increases the allergic response to the model allergen ovalbumin (OVA) in a mouse model.

Objective In the present study, we wanted to confirm the adjuvant effect of another β -1,3-glucan, MacroGard[®] (MG) from baker's yeast in this model. More importantly, we wished to explore the putative effects of extracts from the moulds *Cladosporium herbarum* (CH) and *Penicillium chrysogenum* (PC) using the very same model as used to explore effects of β -glucans.

Methods Groups of eight Balb/c mice were injected with OVA alone, OVA+extract or OVA+MG, into one footpad. On day 21, all mice were reinjected with OVA, before exsanguination on day 26. The levels of OVA-specific IgE, IgG1 and IgG2a in serum were measured by ELISA.

Results Compared with OVA alone, OVA+MG, OVA+CH extract and OVA+PC extract increased OVA-specific IgE and IgG1 levels significantly. For all groups, the levels of IgG2a anti-OVA remained similar to those of the OVA-alone group.

Conclusions Our results show that extracts from CH and PC, and the β -1,3/1,6-glucan from baker's yeast have adjuvant effects on the allergic response in mice.

Keywords adjuvant effect, *Cladosporium herbarum*, β -glucan, mould, *Penicillium chrysogenum*

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Introduction

Mould growth is a common problem in moisture- and water-damaged building constructions [1, 2]. Moulds reproduce by releasing spores (conidia) into the air and a majority of the conidia are between 2 and 20 μ m in diameter and therefore inhalable [2]. Conidia from different moulds contain a number of different allergens, and their capacity to induce an immunological IgE-response is well known [2–4]. One review article suggests that 20–30% of atopic individuals and up to 6% of the general population have respiratory allergy to moulds [5]. As moulds may induce specific allergic reactions in susceptible persons, moulds in indoor environment are also associated with increased prevalence of respiratory symptoms such as asthma and bronchitis [5].

Cladosporium herbarum (CH) is a mould that mainly grows outdoors, but it will contaminate indoor air as well as being a secondary wall colonizer in damp buildings [6]. CH spores are a source of fungal inhalant allergens and three major allergens have been purified and characterized [5]. Sensitiza-

tion to *Cladosporium* and also *Penicillium* is found to be age-dependent and declines rapidly with increasing age (until the age of 20) [7]. Furthermore, it has been observed that children suffering from asthma are more likely to live in homes with significantly greater spore counts of *Cladosporium* species compared with healthy children [8].

Penicillium chrysogenum (PC) is a major indoor fungus [9] regarded as one of the primary wall colonizers in water-damaged dwellings [6]. *Penicillium* species appear to be strongly correlated with sick building syndrome [10, 11], which is a commonly used term for respiratory symptoms in inhabitants of dwellings with poor indoor air quality. Previously, it has been found that bronchial challenging with conidia of *Penicillium* species in sensitized subjects induced an immediate and delayed type of asthma [12].

β -glucans are glucose polymers present as structural constituents in the cell wall of some bacteria, plants, yeast and fungi. The effects of β -glucans on the immune system include anti-tumour [13] and anti-infective [14–16] activities in animals. Furthermore, β -glucans have harmful effects on respiratory health. It has been shown that exposure to β -1,3-glucan gives symptoms of airway inflammation in an experimental study [17]. This observation is supported by a study in which exposure to β -1,3-glucan increased eosinophilic infiltration and prevented allergen-specific IgE down-regulation in response to an inhaled allergen in mice [18].

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In a previous study, we have found that β -glucans isolated from barley and the fungus *Sclerotinia sclerotiorum* significantly increased the IgE and IgG1, but not the IgG2a allergen-specific immune responses in mice [19]. This indicates that β -glucans enhance a Th2-dependent allergic immune reaction in mice. The aim of the present study was to examine the effects of moulds on the development of allergy, using the popliteal lymph node (PLN) assay. The allergen and putative adjuvants were injected subcutaneously (s.c.) into the right hind footpad of the mouse. The systemic and local immune responses were measured in serum (IgE, IgG1 and IgG2a) and the PLN (weight and cell number), respectively. Since moulds contain β -glucan and we previously have found adjuvant activity of the fungus *S. sclerotiorum* [19], we wished to explore similar effects of mould extracts in the same mouse model for allergy. Moreover, by using another β -glucan, MacroGard[®] (MG) from baker's yeast, we wanted to confirm the effects of β -glucans shown previously and also compare the effect of mould with those of β -glucans.

Materials and methods

Animals

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

Preparations for immunization

CH (product no. 1034, Allergon AB, Angelholm, Sweden) and PC (product no. 1084, Allergon AB) were made by adding 10 mL phosphate-buffered saline (PBS) to 0.5 g of mould. The suspension was mixed by gently shaking for 48 h at 4 °C and centrifuged for 20 min at $20\,200 \times g$. The supernatant was dialysed with Spectrapor membrane (MWCO 3500, Spectrum[®] laboratories INC, Rancho Dominguez, CA, USA) against distilled water for 48 h. Water was changed once. The solution was filtered through a sterile filter (pore size 0.2 μ m) and freeze-dried. The immunization doses used were 400, 200, 100, 50, 25 or 12.5 μ g of CH or PC alone or together with 10 μ g OVA (cat. no. A7641, Sigma Chemical Co., St Louis, MO, USA) in PBS/5% with Balb/c serum. The volume injected was 20 μ L consistently.

The immunization doses of the β -1,3/1,6-glucan, MG purified from baker's yeast (Lot no. 440–8, BioTec ASA, Tromsø, Norway) used in the different mice groups, were 100 and 10 μ g of MG alone or together with 50 μ g OVA dissolved in Hank's balanced salt solution (HBSS, Gibco BRL, Paisley, Scotland) with 5% Balb/c serum.

Measurement of protein concentration in mould extracts

The protein content in the extracts were quantified by Bicinchoninic acid assay (BCA[™] Protein Assay Kit, Pierce, Rockford, IL, USA) and further investigated by SDS-PAGE.

SDS-PAGE was performed with the Pharmacia system for horizontal electrophoresis in a Multifor II electrophoresis unit 2117 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with precast polyacrylamid gels (ExcelGel SDS gradient 8-18, Amersham Pharmacia Biotech, Uppsala, Sweden) that were diffusion blotted according to the method by Olsen and Wiker [20]. PC, CH and MG were diluted in sample buffer without reducing conditions, and applied at 15.6 μ g per lane. The gel was blotted on a NitroBind Nitrocellulose transfer membrane (MSI Westboro, MA, USA) for 30 min and stained with Silver staining kit Protein (Amersham Pharmacia Biotech).

Assay for detection of non-reducing glucose and α -mannosyl residues in mould extracts

One milligram Concavalin A (Con A, Amersham Pharmacia Biotech) diluted in 1 mL of 0.1 M Na₂CO₃ with 0.5 M of NaCl, pH 8.3 were biotinylated by incubating with 200 μ L of 1 mg/mL biotin-X-NHS (Calbiochem, San Diego, CA, USA) in dimethylsulphoxide (DMSO, Sigma, St Louis, MO, USA) for 2 h, and then dialysed with Spectrapor membrane (Spectrum[®] Labs Inc., Rancho Dominguez, CA, USA; MWCO 3500) overnight against PBS, pH 7.5 with 0.02 NaN₃ (PBS/azide).

The blot was blocked with PBS/azide with 2% bovine serum albumin (BSA, Biotest AG, Dreieich, Germany) and 1% of hipure liquid gelatin (Norland Products Inc, New Brunswick, NJ, USA) for 2 h, washed for 30 min with PBS/azide with 0.02% Tween (PBS/azide/Tw), incubated with 1 μ g/mL biotinylated Con A diluted in PBS with 0.2% BSA and 0.1% gelatin (PBS/BSA/gelatin) for 2 h in room temperature and overnight at 4 °C. Then, the blots were washed, incubated with alkaline phosphate-conjugated avidin (DakoCytomation, Glostrup, Denmark) diluted 1 : 10 000 in PBS/BSA/gelatin for 2 h, washed, developed with Western lightning CDP-star chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA, USA) and analysed by Kodak Image Station 1000 (Eastman Kodak company, Rochester, NY, USA).

Experimental design

Mice were bled prior to each experiment. On day 0, groups of eight mice were injected s.c. with 20 μ L of one of the following preparations: CH+OVA, CH alone, PC+OVA, PC alone, MG+OVA, MG alone, OVA alone, medium alone or none (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, Ireland). On day 21, the mice were boosted with OVA alone into the same footpad. At the end of experiments (day 26), all the mice were anesthetized with CO₂ and exsanguinated by heart puncture, and their serum level of Ig anti-OVA were measured. There was no OVA contamination of medium, CH, PC or MG as demonstrated by lack of Ig anti-OVA response in mice given each of these reagents alone (data not shown). In the experiments with CH and MG, the PLN from both the left and right hind legs were removed and stored in HBSS (Gibco BRL) until they were stripped of fat and weighed by using

Sartorius Research MC1 (Sartorius, Göttingen, Germany). Each lymph node was then placed in 200 µL HBSS on a Petri dish and teased into a single cell suspension using 18-G needles. Cell suspensions were enumerated using a Coulter Counter Z1 (Beckman Coulter Inc., Miami, FL, USA). To measure the local cellular response, weight and cell number indices were determined by dividing the value of the right lymph node (inoculated) with the value of the left lymph node (control).

Collection of blood samples

Blood samples were drawn from the distal part of the saphenous vein [21, 22]. After removing hair from the area with a scalpel blade, the vein was punctured with a 21-G needle and 200 µL of blood collected into heparinized capillary tubes (Vitrex, Herlev, Denmark). Serum from all blood samples were prepared and stored at -20°C until analysed.

Assay for measurement of IgG2a anti-OVA by ELISA

Polystyrene microtitre plates (Immulon II M 129B, Dynatech Laboratories, Chantilly, VA, USA) were coated with 100 µL of 2 µg/mL OVA diluted in 0.05 M carbonate/bicarbonate buffer pH 9.6. The coated plates were incubated for 1 h at room temperature and then overnight at 4°C . After washing with 0.1 M Tris-HCl buffer pH 7.4, containing 0.05% Tween 20 (Tris/Tw), the plates were blocked with 100 µL Tris/Tw with 1% BSA. After 1 h at room temperature the plates were washed Tris/Tw. To the plates were then added 100 µL of OVA-specific standard IgG2a mouse serum (standard curve) diluted in BSA/Tris/Tw 4-folds from 1 : 50 in triplicate. Then 100 µL of the sera diluted in BSA/Tris/Tw 1 : 10 was added in duplicate. Normal sera were added to two wells as negative control and sera from OVA-boosted mice were added to two wells as a positive control. To control the background, BSA/Tris/Tw was added to three wells. The plates were incubated for 2 h and then overnight at 4°C . The plates were washed five times with Tris/Tw and incubated with 100 µL of anti-mouse IgG2a (Cat. no. 02012D-553388, BD Biosciences Pharmingen, San Diego, CA, USA) diluted 1 : 500 in Tris/Tw for 2 h at room temperature. After five new washes, the plates were incubated for 1 h at room temperature with streptavidin in complex with biotinylated alkaline phosphatase (ABCComplex/AP, DAKO, Glostrup, Denmark) diluted 1 : 50 in 0.05 M Tris/HCl, pH 7.6. The plates were washed five times again, and then 100 µL of *p*-nitrophenyl phosphate disodium hexahydrate diluted in 10% diethanolamine buffer, pH 9.8 was added. Optimal density (OD) was measured at 405 nm in an MRX Microplate Reader (Dynatech Laboratories, Chantilly, VA, USA) connected to a PC using BioLinx software (Dynatech Laboratories) for calculations.

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

The procedures have been described in detail elsewhere [19, 23]. In short, to detect IgE anti-OVA, microtitre plates were coated with rat anti-mouse IgE antibody (clone LOME-3, Serotec, Oxford, UK) and incubated subsequently with biotinylated OVA, streptavidin in complex with biotinylated

alkaline phosphatase (ABCComplex/AP, DAKO) and lastly *p*-nitrophenyl phosphate disodium hexahydrate diluted in diethanolamine buffer. Standard curves of a mouse serum pool containing defined amounts of IgE anti-OVA were included on each plate. OD was measured as described above. Measurement of IgG1 was carried out 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).

Assay for measurement of β -glucan and endotoxin

The amount of β -glucan was determined by a commercially available *Limulus Amebocyte Lysate* (LAL) test for β -glucan (catalogue number GS001, Cape Cod, Falmouth, MA, USA). The amount of endotoxin was determined by another LAL test for endotoxin (product number N283-125, Bio Whittaker, Walkersville, MD, USA).

Statistical analysis

ANOVA on Ranks was used to compare all groups, followed by a pairwise Mann-Whitney Rank Sum test to compare differences between OVA and treated groups. Outlayer values were excluded by Nalimov's test ($P \leq 0.01$). Statistical program used was SigmaStat[®] (Jandel Scientific, Erkrath, Germany). The limit for statistical significance was set at $P = 0.05$.

Results

Mould extracts – protein concentration and yield

The yield after water-extraction of moulds was less than 6% of the total mould material. Extracts of CH and PC contained 5–6% protein as measured with BCA. The low protein content was confirmed by SDS-PAGE, where as much as 250 µg of mould extract had to be loaded to detect protein bands after Coomassie blue staining (data not shown). Silver-stained SDS-PAGE gel loaded with 15.6 µg of mould extract per lane showed a broad range of different sized proteins, in addition to smears in a molecular weight (MW) range of 25 000–100 000 for both CH and PC (Fig. 1). In blotting analyses, Con A bound to both bands and smears (data not shown). Neither bands nor smears were seen on gel (Fig. 1) or blot with the same amount of MG applied.

Endotoxin and β -glucan in test samples

The levels of endotoxin in CH, PC, MG and medium were below that of the OVA solution (~ 400 EU/mL). Thus, the adjuvant effects observed cannot be caused by endotoxin. The β -1,3-glucan content was 1.0% and 1.4% w/w of the water-soluble CH and PC extracts, respectively.

Effects of CH on the systemic anti-OVA response and local PLN response

CH (12.5 µg)+OVA (10 µg) enhanced the serum levels of IgE and IgG1 anti-OVA more than 3-fold ($P \leq 0.001$) compared with OVA alone (Fig. 2). CH (400 µg)+OVA increased the

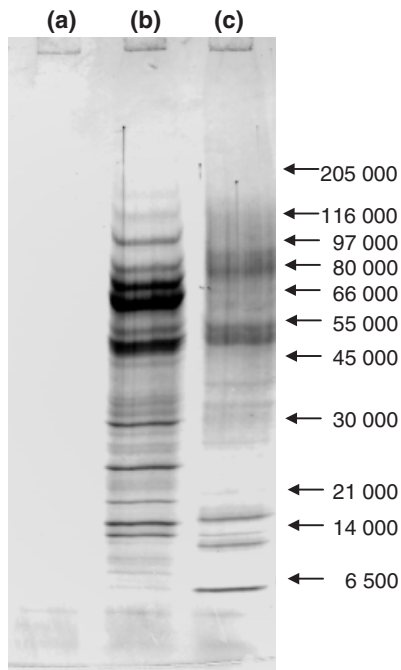


Fig. 1. Silver-stained SDS-PAGE gel with 15.6 µg MacroGard® (MG) (a), *Penicillium chrysogenum* (PC) (b) and *Cladosporium herbarum* (CH) (c) extracts per lane. Arrows with numbers indicate the sizes of MW standard. Note the smears in the molecular weight (MW) range 25 000–100 000.

production of OVA-specific IgE 3-fold ($P = 0.001$), but not IgG1, compared with OVA alone. However, CH concentrations of 25–200 µg+OVA did not increase the levels of IgE and IgG1 specific anti-OVA compared with OVA alone. The levels of OVA-specific IgG2a did not vary significantly among the groups (Table 1a).

The weight of PLN in all groups treated with CH+OVA increased up to 1.7-fold ($P \leq 0.02$) compared with the OVA group (data not shown). However, the cell number in the CH+OVA treated groups did not differ from the OVA group. In a corresponding control experiment a significant increase in cell number, but not PLN weight, was found in groups treated with CH+OVA compared with OVA alone (data not shown).

Effects of PC on the systemic anti-OVA response

Mice given PC (12.5 µg)+OVA increased the anti-OVA IgE levels in serum more than 20-fold compared with the OVA group ($P < 0.001$) (Fig. 3a). At higher doses of PC together with OVA, the levels of anti-OVA IgE were not that high, but still significantly increased (2–5-fold) ($P \leq 0.038$). A similar increase was observed for anti-OVA IgG1 compared with OVA alone (Fig. 3b). At PC doses of 12.5, 25 and 50 µg together with OVA, the anti-OVA IgG1 responses increased 14-fold ($P = 0.001$), 4-fold ($P = 0.014$) and 3-fold ($P = 0.029$), respectively. Higher doses of PC (100, 200 or 400 µg) given together with OVA did not give any increase in the anti-OVA IgG1 levels. For all groups, the levels of OVA-specific IgG2a were low and not statistically higher than those of the OVA group (Table 1a). At day 20, the OVA-specific IgE response

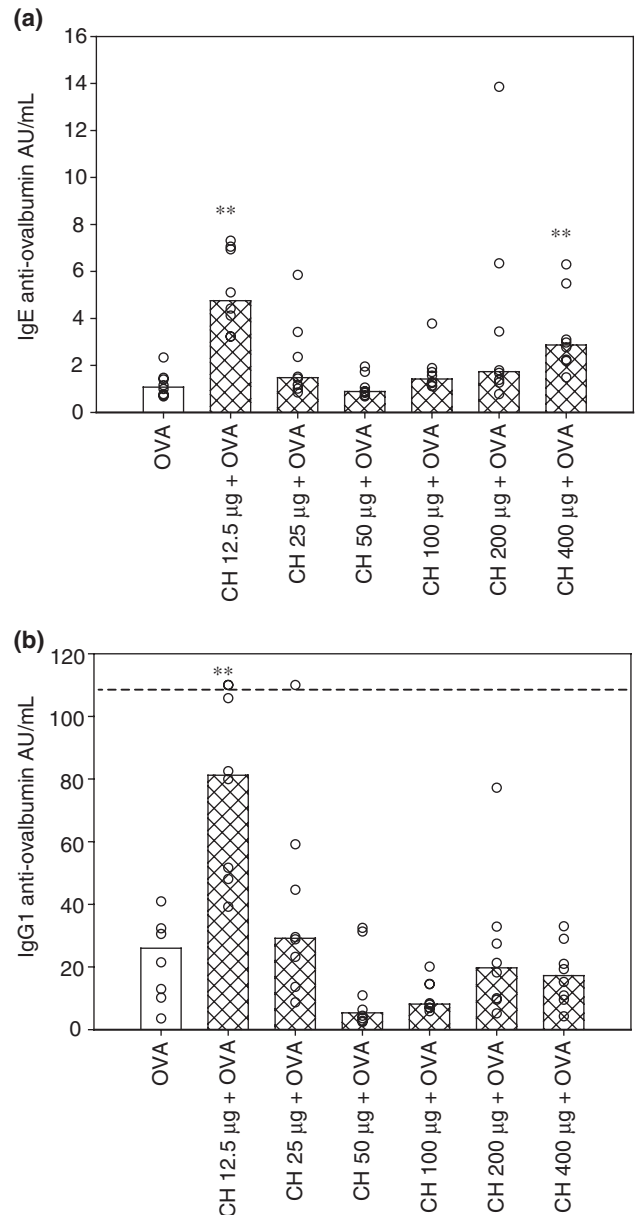


Fig. 2. Levels of ovalbumin (OVA)-specific IgE (a) and IgG1 (b) measured in mouse serum day 26. Mice were injected with *Cladosporium herbarum* (CH)+OVA or OVA on day 0. Doses of CH were: 12.5, 25, 50, 100, 200 and 400 µg given together with 10 µg of OVA, which also was given alone. At day 21, all mice were boosted with 10 µg of OVA. Values are given in arbitrary units (AU) measured for individual mouse (circle) and median for groups of eight mice (columns). Dotted lines show upper detection limits for IgG1 (110 AU/mL) (* $P < 0.05$ and ** $P \leq 0.001$ compared with OVA alone).

was, as usual in this set-up, low in all groups, indicating a low primary response.

Effects of MG on the systemic anti-OVA response and local PLN response

MG (100 µg)+OVA increased the levels of OVA-specific serum IgE and IgG1 9- and 7-fold, respectively, compared with OVA alone ($P < 0.001$) (Fig. 4). This was confirmed in a similar experiment (data not shown). Although the rise was not statistically significant compared with OVA alone, the levels of anti-OVA IgE and IgG1 tended to rise 2–3-fold in

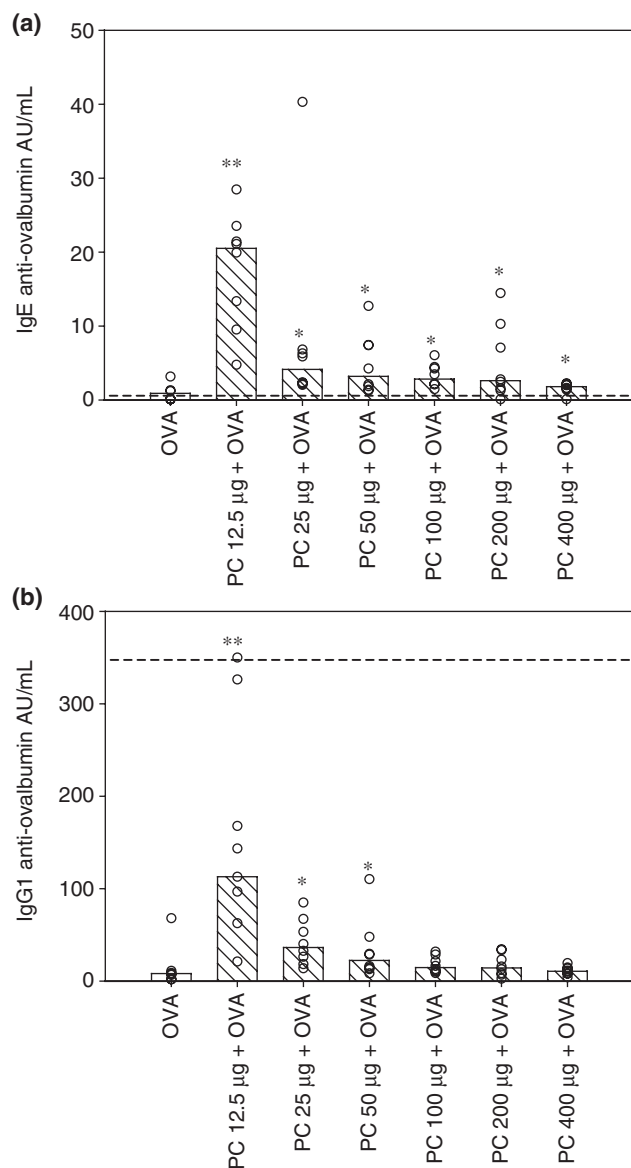


Fig. 3. Levels of ovalbumin (OVA)-specific IgE (a) and IgG1 (b) measured in mouse serum day 26. Mice were injected with *Penicillium chrysogenum* (PC)+OVA or OVA on day 0. Doses of PC were: 12.5, 25, 50, 100, 200 and 400 µg given together with 10 µg of OVA, which also was given alone. At day 21, all mice were boosted with 10 µg of OVA. Values are given in arbitrary units (AU) measured for individual mice (circle) and median for groups of eight mice (columns). Dotted lines show upper and lower detection limits for IgE (350 AU/mL as upper) and IgG1 (0.1 AU/mL as lower) (* $P < 0.05$ and ** $P \geq 0.001$ compared with OVA alone).

serum of mice injected with MG (10 µg)+OVA. For all groups, the levels of OVA-specific IgG2a were low and not statistically higher than the OVA group (Table 1b). The Ig responses at day 0 were as expected, low. The weight and cell number indices of groups giving MG+OVA were not significantly different from those given OVA alone (data not shown).

Discussion

To test the present hypothesis that moulds have adjuvant effects on allergic immune responses, an s.c. administration

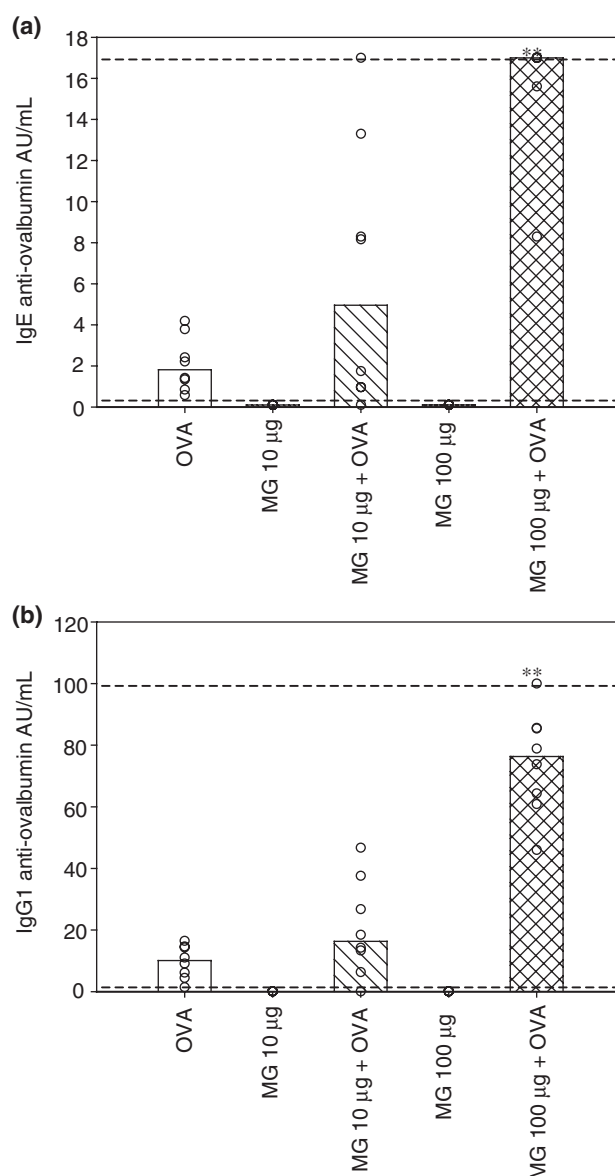


Fig. 4. Levels of ovalbumin (OVA)-specific IgE (a) and IgG1 (b) measured in mouse serum day 26. Mice were injected with MacroGard® (MG)+OVA, MG or OVA on day 0. Doses of MG were: 10 or 100 µg given alone or together with 50 µg OVA, which also was given alone. Day 21 all mice were boosted with 50 µg OVA. Values are given in arbitrary units (AU) measured for individual mouse (circle) and median for groups of eight mice (columns). Dotted lines show upper and lower detection limits for IgE (0.1 and 17 AU/mL) and IgG1 (0.1 and 100 AU/mL) (* $P < 0.05$ and ** $P \leq 0.001$ compared with OVA alone).

route was chosen. In the respiratory administration procedure, it is difficult to control the dose given. Whereas administration by the respiratory system mainly favours a Th2-dependent response [24], a preferential Th1-type environment exists in peripheral lymph nodes like PLN [25]. To test our hypothesis, we have chosen a less Th2-favourable route of administration and still found a highly adjuvant effect of moulds. However, in order to get final answers, it is important to administer these mould extracts by the airways, because this is the most relevant exposure route.

In our daily life we are mainly exposed to conidia [26], whereas most commercially available moulds, like ours,

Table 1. Significant decrease (Yes) or no difference (No) compared with OVA, median and 25% and 75% percentile of OVA-specific IgG2a levels measured in mouse serum on day 26 for groups immunized day 0 with CH+OVA, PC+OVA, MG+OVA or OVA

	CH			PC		
	Significant change*	Median	25% and 75% percentile	Significant change*	Median	25% and 75% percentile
(a)						
OVA		6.44	6.1–8.7		7.33	6.2–9.5
12.5 µg+OVA	No	5.17	4.0–6.4	No	7.09	5.7–23.0
25 µg+OVA	No	3.76	3.1–5.8	No	7.94	6.2–10.6
50 µg+OVA	No	6.17	5.2–7.3	Yes	5.52	4.9–6.2
100 µg+OVA	No	4.93	4.4–8.1	Yes	5.25	4.7–5.5
200 µg+OVA	No	6.72	5.1–8.9	No	5.68	4.8–7.4
400 µg+OVA	No	6.16	4.4–6.9	No	6.45	5.3–6.8
	MG					
	Significant change*	Median		25% and 75% percentile		
(b)						
OVA		36.72		31.6–40.6		
10 µg	Yes	28.06		26.3–29.4		
10 µg+OVA	No	29.97		27.2–54.0		
100 µg	No	33.49		33.2–36.9		
100 µg+OVA	No	45.63		39.3–64.4		

Doses of CH (a) and PC (a) were: 12.5, 25, 50, 100, 200 and 400 µg given together with 10 µg OVA, and dose of OVA alone (10 µg). Doses of MG (b) was 10 or 100 µg and was given alone or together with 50 µg OVA, which also was given alone. At day 21, all mice were boosted with 10 or 50 µg OVA. Values are given in arbitrary units (AU). * $P \leq 0.05$.

OVA, ovalbumin; CH, *Cladosporium herbarum*; PC, *Penicillium chrysogenum*; MG, MacroGard®.

consist of mycelium and little conidia. On the other hand, since conidia are developed from mycelium, one may assume that most compounds are present in both parts of the mould. This is supported by a previous study in which it was found that antigens in mycelium from *Alternaria alternata* were all present in the conidia [26]. Furthermore, our extracts are water-soluble, and the water-soluble fraction of the mould will more easily dissolve in the mucous of the airways. Thus this fraction, as compared to the water-insoluble one, most probably enters the body more efficiently. One can therefore speculate that the soluble fraction has a greater effect on the respiratory tract than the insoluble one. Moreover, approximately 5% w/w of the natural mould was extracted, and these extracts consisted of 5–6% w/w of proteins. In SDS-PAGE a broad-sized range of bands implied a complex composition of proteins. Most bands and all smears were, in a broad MW range, seen in both gel and blot analysed with Con A. This indicated that the extracts contained carbohydrates and/or glycoproteins, as Con A binds to non-reduced glucose and α -mannosyl residues.

For both mould extracts, the adjuvant effect was most pronounced at the lowest dose examined. A greater response at lower, than higher doses, is a phenomenon that has been observed in previous field studies on cat allergen and allergic symptoms [27] and in toxicological experiments [28, 29]. Provided that our findings in mice can be extrapolated to humans, our findings suggest that exposure to moulds in the environment have an effect on the development of allergic diseases even though the mould infestation in the building is moderate and even invisible. The β -1,3-glucan content of CH and PC extracts was less than 2% w/w. Provided that β -1,3-glucan is the only compound with a potent adjuvant effect at

the lowest dose of the extracts, this β -1,3-glucan would be 600–800 times or more effective than MG. However, it is more likely that moulds contain other adjuvants in addition to β -glucan and that the action of these substances is somehow weaker at higher doses. It would have been interesting to see whether the adjuvant effect at low doses as well as the potential for blocking of the responses could have been diluted out.

At the highest dose of CH, the β -1,3-glucan content (4 µg) was only 1/25 of the MG dose (100 µg) needed for a significant response (Fig. 4). Since it has been shown that the immunological activity of β -1,3-glucan depends on its molecular weight and chain branching [30], one can speculate that the immunological activity of the β -1,3-glucan in the CH extract is more potent than the MG β -1,3-glucan and therefore gives an adjuvant effect at our highest CH dose. Unlike the mould extracts, the β -1,3-glucan MG gave the most pronounced response at the highest dose. In a previous study using the same PLN assay, β -1,3-glucans from the fungus *S. sclerotiorum* and from barley, given together with OVA [19], had a similar adjuvant effect as that observed with MG+OVA. Since β -1,3-glucans give an increasing adjuvant effect with increasing concentrations, the effect seen with 400 µg of CH is most likely caused by β -1,3-glucan alone or in addition to other compounds. As the effect observed with concentrations of PC extract higher than 12 µg was similar in magnitude to the effect with 400 µg of CH, a β -1,3-glucan may also be partly responsible for the adjuvant activity observed with higher PC concentrations.

Since 12.5 µg of CH and PC contains as low concentrations of β -1,3-glucan as 0.1–0.2 µg, it is unlikely that a β -1,3-glucan alone was responsible for the high adjuvant activity observed.

This, taken together with the U-shaped dose-response curve for CH, suggests that different compounds were responsible for the effects observed of the high and the low doses of mould extract, respectively. Furthermore, we exclude the possibility that endotoxin caused any of the adjuvant effects because the concentrations of endotoxin in the extracts were found to be lower than in OVA.

Development of possible allergies against PC and CH per se was not examined in our experiment. In this study, we examined the mould as a provoker of allergic diseases. PC was found to be a four to five times more potent Th2-adjuvant than CH in our set-up. These findings, together with the fact that PC, in contrast with CH, is mainly growing indoors, indicate that PC is a more potent provoker in causing allergic diseases than CH.

Effects related to indoor air are associated with non-specific airways inflammation in humans [31]. We wanted to examine the effect of moulds in a model for local inflammation and used the PLN assay. Responses on PLN weight and cell number were noted and increased responses in one of the two parameters were found in each experiment. Since PLN weight and cell numbers are similar parameters, an increase in one of them should suffice as a local cellular response. Consequently, we assume that PC and CH have an effect on the local cellular responses in our experiment.

We have shown that CH and PC clearly provide OVA-specific immune response in a Th2-dependent manner. PC also caused a more potent adjuvant effect than CH. Moreover, in this study we found that the dose of mould is highly relevant and that lower doses of mould may be more potent than higher doses. Since the mould extracts contained less than 2% β -1,3-glucan, the above observation suggests that mould also contains other and more potent adjuvants than β -1,3-glucan that are active in lower concentrations. However, further studies on moulds are warranted to find out how they affect us in real life and whether they enhance allergy. Our observations suggest that living in an environment with moulds can aggregate not only specific allergy to mould allergens but also increase allergy to other environmental allergens.

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