

Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* in murine models of allergy

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Summary

Background Previous studies have demonstrated that chitin in the form of microparticles that can be phagocytosed is a potent macrophage stimulator and promotes a Th1 cytokine response and it has been shown that oral administration of chitin microparticles is effective in down-regulating serum IgE and lung eosinophilia in a mouse model of ragweed allergy. To date there have been no studies on the effectiveness of directly applying chitin microparticles to the respiratory tract as a treatment for allergic symptoms.

Objective To test the effectiveness of chitin microparticles when given intranasally as a treatment for the symptoms of respiratory allergy and allergic asthma and to compare its effectiveness in two different mouse models of allergy, namely to *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus*.

Results The intranasal application of microgram doses of chitin microparticles is an effective treatment for reducing serum IgE and peripheral blood eosinophilia, airway hyper-responsiveness and lung inflammation in both allergy models results in elevation in Th1 cytokines IL-12, IFN- γ and TNF- α and reduction in IL-4 production during allergen challenge.

Conclusion Chitin microparticle suspensions have Th1 immunostimulatory properties and are effective when administered intranasally in mice. The stimulation of the nasal associated lymphoid tissue with chitin microparticles could offer a novel and natural approach to treating allergic disease in humans.

Keywords allergy, *Aspergillus fumigatus*, chitin, Der p, IFN- γ , IL-12, immunotherapy, intranasal treatment

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Introduction

The house dust mite, *Dermatophagoides pteronyssinus* (Der p), is one of the leading causes of allergic asthma and treatment strategies are of considerable importance in industrialized countries [1–3]. *Aspergillus fumigatus* is a ubiquitous fungus in agricultural environments and inhalation of spores can result in a variety of hypersensitivity diseases including asthma, hypersensitivity pneumonitis and allergic bronchopulmonary aspergillosis (ABPA) [4, 5]. Corticosteroids are commonly used to reduce inflammation, while effectively improving lung function and airway hyper-responsiveness [6]. However, the long-term use of steroids has undesirable side-effects and there is a need for alternative treatments. An alternative approach is to modulate the Th1/Th2 lymphocyte populations and re-direct the immune response away from a Th2, IgE-mediated allergic

hypersensitivity reaction towards the more favourable Th1 response [7–9]. Cytokines of primary importance in modulating the Th1/Th2 balance are IL-12 and IFN- γ and there is much research into their therapeutic use [10, 11]. However, there are toxicity and dosage problems with the direct administration of cytokines [12]. Another approach is to use natural up-regulators to elevate endogenous IL-12 or IFN- γ . Many microbial products, including heat-killed bacteria and CpG motifs, up-regulate Th1 cytokines [13, 14] but there is a concern that such microbial products could overstimulate the Th1 cytokine profile leading to Th1-mediated pathology such as autoimmunity [15]. We propose an alternative to microbial products, namely chitin microparticles (CMP). Chitin (a natural polysaccharide of *N*-acetyl-D-glucosamine), in the form of microparticles (1–20 μ m in diameter) is an interesting candidate for immunomodulation because it can be derived from non-microbial sources such as shrimp, crab and lobster. It is non-allergenic, biodegradable and biocompatible, and chitin products are already in wide use in the medical, veterinary, cosmetic, health supplement and environmental industries [16]. Chitin would be expected to induce a Th1 response because

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it is also a major component of fungal spores and the innate immune system of the lung is well adapted for the phagocytic clearance of airborne spores, which is driven by Th1 cytokines [17]. The macrophage is the most abundant leucocyte in the mucosal layer of the nasal mucosa and respiratory tract and is responsible for phagocytic clearance of microbes and particulates and the secretion of IL-12, IL-18 and TNF- α cytokines that promote an effective cell-mediated immune response to inhaled particulates [18–20]. These cytokines induce IFN- γ production by natural killer (NK) cells and Th1 lymphocytes. IFN- γ acts synergistically with the macrophage-derived cytokines to promote a Th1 cell-mediated immune response and also down-regulates the production of Th2 cytokines, including the Th2 cytokines IL-4 and IL-5 [21, 22].

The aim of the present study is to show that treatment of sensitized mice with as little as four to five daily doses of microgram quantities of chitin microparticles (CMP) given intranasally is an effective treatment for reducing allergic symptoms provoked by allergen challenge, and that CMP treatment is sufficient to significantly reduce serum IgE and lung eosinophilia in mice. This study also investigates the effects of treatment with CMP on the levels of IL-12, IFN- γ , TNF- α and IL-4 to determine if CMP can promote Th1 cytokines, which are known to be of great importance in reducing antigen-induced airway hyper-responsiveness [11, 23].

Materials and methods

Chitin microparticle suspension preparation

CMP were prepared from pure chitin (Sigma-Aldrich, Poole, UK) by sonication of a suspension of chitin in sterile, endotoxin-free phosphate-buffered saline (PBS). The particles were collected by centrifugation, washed with 20% (v/v) ethanol to ensure sterility and washed five times with sterile PBS to remove soluble chitin. The suspension of CMP was examined by flow cytometry (FACS) and compared with 1- μ m and 20- μ m standardized beads (Polysciences, Inc., Warrington, PA, USA). Ninety-eight per cent of the particles were smaller than 20 μ m and 33% were less than 1 μ m in size. Sterility was confirmed by absence of colony-forming units after plating an aliquot on an agar plate. Endotoxin was measured by the Limulus Amebocyte Lysate Assay (BioWhittaker, Wokingham, UK) and shown to be < 1 EU/mL.

Allergen extracts

Standardized *D. pteronyssinus* (Der p) extract (Greer Laboratories, Lenoir, NC, USA) containing 10 000 allergy units (AU)/mL was diluted into sterile endotoxin free PBS.

A. fumigatus (Afu) allergen extract from a 1-week culture filtrate was prepared as described by Arruda et al. [24]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed a major 18 kDa band. N-terminal sequencing identified this as Asp f1.

Sensitization

Female C57BL/6 mice were given 4-weekly intraperitoneal (i.p.) injections of a mixture of allergen extract (63 AU Der p; 200 μ g Afu) with alum (2 mg) in 100 μ L of sterile PBS.

Allergen challenge and treatment with CMP

Five days after the last i.p. injection, sensitized mice were anaesthetized with isoflurane and challenged with 50 AU of Der p extract, or 10 μ g Afu allergen extract in PBS given intranasally followed by intranasal doses of PBS or CMP or a particulate control (pc) of 1- μ m polystyrene beads in 50 μ L given 1 h later. In a separate experiment it was shown that approximately 50% of fluorescein isothiocyanate (FITC)-labelled microbeads given intranasally could be recovered from the lungs after 30 min. Allergen challenge and treatment were repeated on a daily basis as indicated in the Results. Pilot studies indicated that maximal changes in the parameters measured in this study could be observed after a minimum of three daily challenge/treatments and three to five daily treatments were used.

Peripheral blood eosinophils

Blood was collected from the tail vein of mice for estimation of peripheral eosinophils. The total leucocytes were counted with an automatic cell counter and the proportion of eosinophils determined by differential counting of May-Grünwald-Giemsa-stained blood smears. Results are expressed as 10^6 cells/mL.

Serum IgE and Afu-specific IgG1

Total serum IgE was measured by sandwich enzyme-linked immunosorbent assay (ELISA; BD PharMingen, Cowley, UK) in blood serially diluted from a maximum dilution of 1 : 20 to give values that were linear with respect to a standard curve of mouse IgE. Results are expressed in μ g/mL. Afu-specific IgG1 was measured by ELISA using 96-well plates coated with Afu allergen extract. Antibody was detected with horseradish peroxidase (HRP)-labelled anti-mouse IgG1. Results are expressed as relative absorbance units (OD450). Both IgE and IgG1 remained significantly elevated in excess 8 days after allergen challenge.

Intracellular cytokine staining

After treatment, mice were humanely sacrificed by CO₂ asphyxiation and their spleens removed and homogenized in PBS. The homogenate was filtered and red blood cells lysed with ammonium chloride lysing reagent (BD Pharmingen) and fixed with 4% (v/v) paraformaldehyde for 20 min. The cells were washed with PBS supplemented with 3% (v/v) heat-inactivated fetal calf serum with 0.1% (w/v) sodium azide (FSB), re-suspended in 10% (v/v) dimethyl sulfoxide in FSB and stored at -80 °C.

Cells were permeabilized with Cytoperm wash buffer (CPB, BD Biosciences, Cowley, UK) for 15 min at 4 °C and aliquots of 10^6 cells were blocked by incubation for 30 min at 4 °C with CPB supplemented with 50 μ g/mL rat IgG. Intracellular cytokines were stained with 1 μ g R-phycoerythrin (PE)-conjugated rat anti-mouse cytokine monoclonal antibody (BD Biosciences). The clones used were 11B11 (IL-4), C15.6 (IL-12), MP6-XT22 (TNF- α), XMG1.2 (IFN- γ). Staining was performed by incubation for 30 min at 4 °C. The cells were washed with CPB followed by FSB and re-suspended in 0.5 ml FSB.

Flow cytometry was performed with a FACScan flow cytometer (Beckton Dickinson, Mountain View, CA, USA) using CellQuest software. Data were collected for 20 000 cells. The average forward scatter (FSC) of spleen cells was 100 in all

cases. Stained cells (FSC > 100, FL2 > 100) were gated and the proportion of these cells staining intensely for PE (PE > 1000) was calculated. Results are expressed as the percentage intensely stained cells after subtraction of background fluorescence for unstained cells incubated with rat IgG (% PE > 1000). For IL-4, the geometric mean fluorescence (GMF) was measured for stained cells and the background subtracted.

Lung histology

Immediately after treatment, the lungs of mice from each treatment group were fixed in 10% (v/v) neutral buffered formalin and sent for independent analysis. Lungs were embedded in paraffin, sectioned and stained with haematoxylin and eosin. The slides were evaluated for peribronchial inflammation and scores were assigned on a scale of 0–4, corresponding to a score of normal to severe, respectively [25].

Whole-body plethysmography

Airway hyper-responsiveness is known to alter breathing patterns, including an extension of the expiration time. This change can be quantified by the measurement of enhanced expiratory pause (Penh). In this study, airway hyper-responsiveness was measured using unrestrained whole-body plethysmography [26] with a four-chamber system (Buxco, Sharon, CT, USA). Mice were first challenged with intranasal antigen and allowed to recover for 2 h before being placed into the chambers and their breathing monitored for 10 min. When acclimatized, their baseline response was measured for 5 min. The mice were then subjected to 1 min of aerosolized PBS, followed by progressively increasing doses of methacholine (5, 10, 20, 30, 40 mg/mL PBS). Responses are recorded for 5 min in every case with a short interval between to allow return to baseline Penh.

Each group contained four to eight mice. Results are presented as the average percentage elevation in Penh above baseline after a challenge of methacholine.

Statistics

Results are the average for four to eight mice/group and error bars are $\pm$ SEM. Significance was determined by Student's two-tailed *t*-test. Significance was accepted for $P < 0.05$.

Results

Intranasal treatment with CMP-reduced serum IgE and peripheral blood eosinophilia

Sensitized mice ($n = 4$ –8/group) in the Der p (H = Der p) or Afu (A = Afu) allergy models were challenged daily with Der p or Afu allergen extracts, given intranasally, followed 1 h later by intranasal treatment with CMP. Sensitized mice treated with PBS alone (HP, AP) had high serum IgE and peripheral blood eosinophilia in both models. Treatment with five daily doses of 25 μ g CMP (HC), but not a particulate control of 25 μ g 1 μ m polystyrene beads (Hpc), produced a significant decrease of 45% in total serum IgE in the Der p allergy model (Fig. 1a, $P < 0.005$). In the Afu model a significant decrease of 80% in total IgE was also found after five daily doses of 17 μ g CMP (Fig. 1b, AC1 $P < 0.0005$). This effect was maintained when treated mice were re-challenged with Afu extract alone given 1 week later (Fig. 1b, AC2 $P < 0.0005$). Afu-specific IgG1 after five daily doses of 17 μ g CMP was also significantly decreased by 79% (Fig. 1c, AC1 $P < 0.001$). On re-challenge 1 week later, the Afu-specific IgG1 remained significantly lower (Fig. 1c, AC2 $P < 0.01$). Treatment with four intranasal daily doses of 25 μ g CMP decreased peripheral blood eosinophilia by 36% in the Der p model and 58% in the Afu model (Fig. 2, $P < 0.05$). Treatment with four doses of 17 μ g of a particulate control (Hpc) showed no effect in the Der p model.

Intranasal treatment with CMP-elevated IL-12, IFN- γ and TNF- α and decreases IL-4 in allergen-challenged mice

To assess whether intranasal treatment with CMP modulates the production of Th1 cytokines *in vivo* in sensitized mice during allergen challenge, spleens were isolated, homogenized and IL-12, IFN- γ , TNF- α and IL-4 were measured by intracellular cytokine staining. Cytokine-producing activity was assessed by measuring the proportion of intensely stained cells positive for the respective anticytokine antibody labelled with PE. The Th1 cytokine IL-12 was significantly elevated fourfold in the Der p model (Fig. 3a, $P < 0.005$) and 1.7-fold in the Afu model, after intranasal treatment (Fig. 3b). A particulate control (Hpc, Apc) did not induce a significant elevation in IL-12 in either allergy

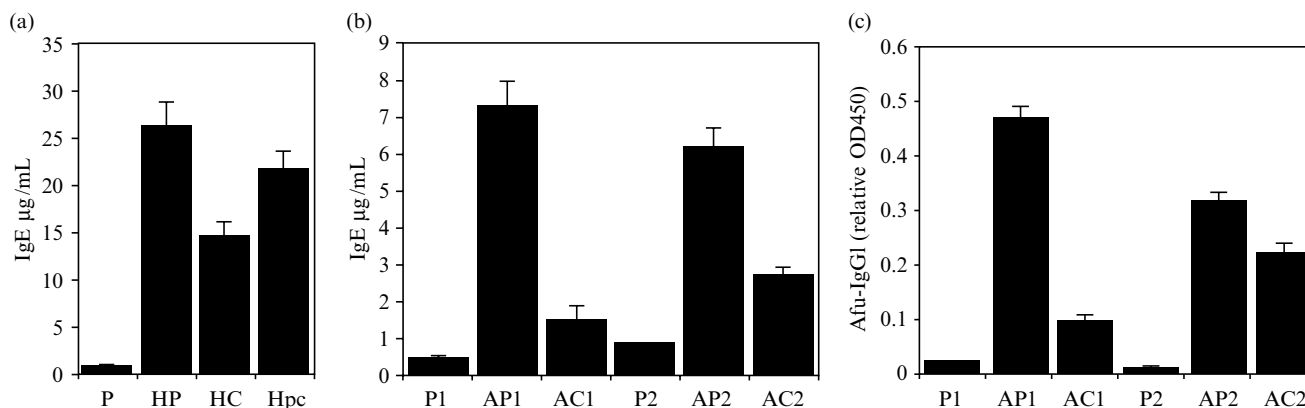


Fig. 1. (a) Effect of intranasal CMP treatment on total serum IgE in the Der p model measured 4 days after treatment with five daily doses of 25 μ g CMP (HC), PBS (HP) or a particulate control (Hpc) given intranasally to sensitized mice after allergen challenge. (b) Effect of intranasal CMP treatment on total serum IgE in the Afu model after treatment with five doses of 17 μ g CMP (AC1) or PBS (AP1) after allergen re-challenge (AP2, AC2) the following week. (c) Afu-specific IgG1 after intranasal treatment with five doses of 17 μ g CMP (AC1), or PBS (AP1) and after re-challenged (AP2, AC2) with allergen the following week. P represents non-sensitized mice treated with PBS.

model when administered in the same way or dose as CMP. IFN- γ was elevated 1.7-fold in the Der p model (Fig. 3c) and 1.3-fold in the Afu model (Fig. 3d, $P < 0.005$). TNF- α was elevated 2.2-fold in the Der p model (Fig. 3e, HC $P < 0.05$)

and 2.4-fold in the Afu model (Fig. 3e, AC $P < 0.05$). Comparison of the GMF of spleen cells stained for the Th2 cytokine IL-4 showed a decrease of 34% in the Der p model and 27% in the Afu model after intranasal treatment with CMP. No decrease was produced with the particulate control tested in the Der p model (Fig. 3f).

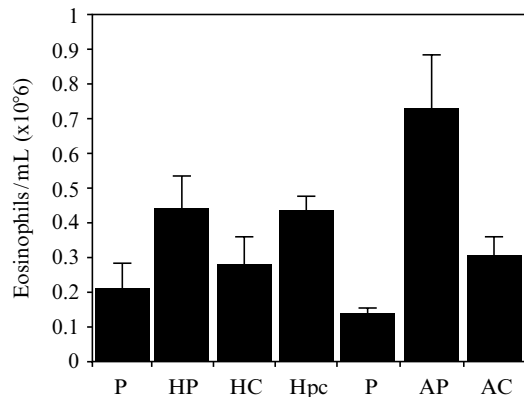


Fig. 2. Effect of intranasal CMP treatment with four daily doses of 20 μ g CMP (HC), PBS (HP), or a particulate control (Hpc) in the Der p model and 17 μ g CMP (AC) or PBS (AP) in the Afu model on peripheral blood eosinophilia. Measurements were made 1 day after treatment. P represents nonsensitized mice treated with PBS.

Airway hyper-responsiveness is reduced by intranasal treatment with CMP

To ascertain if CMP treatment improved the respiratory physiology of allergic mice, airway hyper-responsiveness (AHR) was measured in both allergy models by measuring the change in Penh, a parameter of pulmonary function, which is elevated in asthmatic attacks, of allergen-challenged mice in response to nebulized methacholine. Intranasal CMP treatment of Der p-challenged mice responded with reduced AHR to all concentrations of methacholine tested (Fig. 4a). This improvement was observed after 3 days of an intranasal treatment course of 25 μ g CMP given to sensitized mice that had received allergen challenge 1 h before treatment (Fig. 4b, HC [0] $P < 0.001$) and after re-challenge with allergen alone given 4 days after completion of treatment with a total of four daily doses of 25 μ g CMP preceded by allergen challenge (Fig. 4b, HC [27], $P < 0.001$).

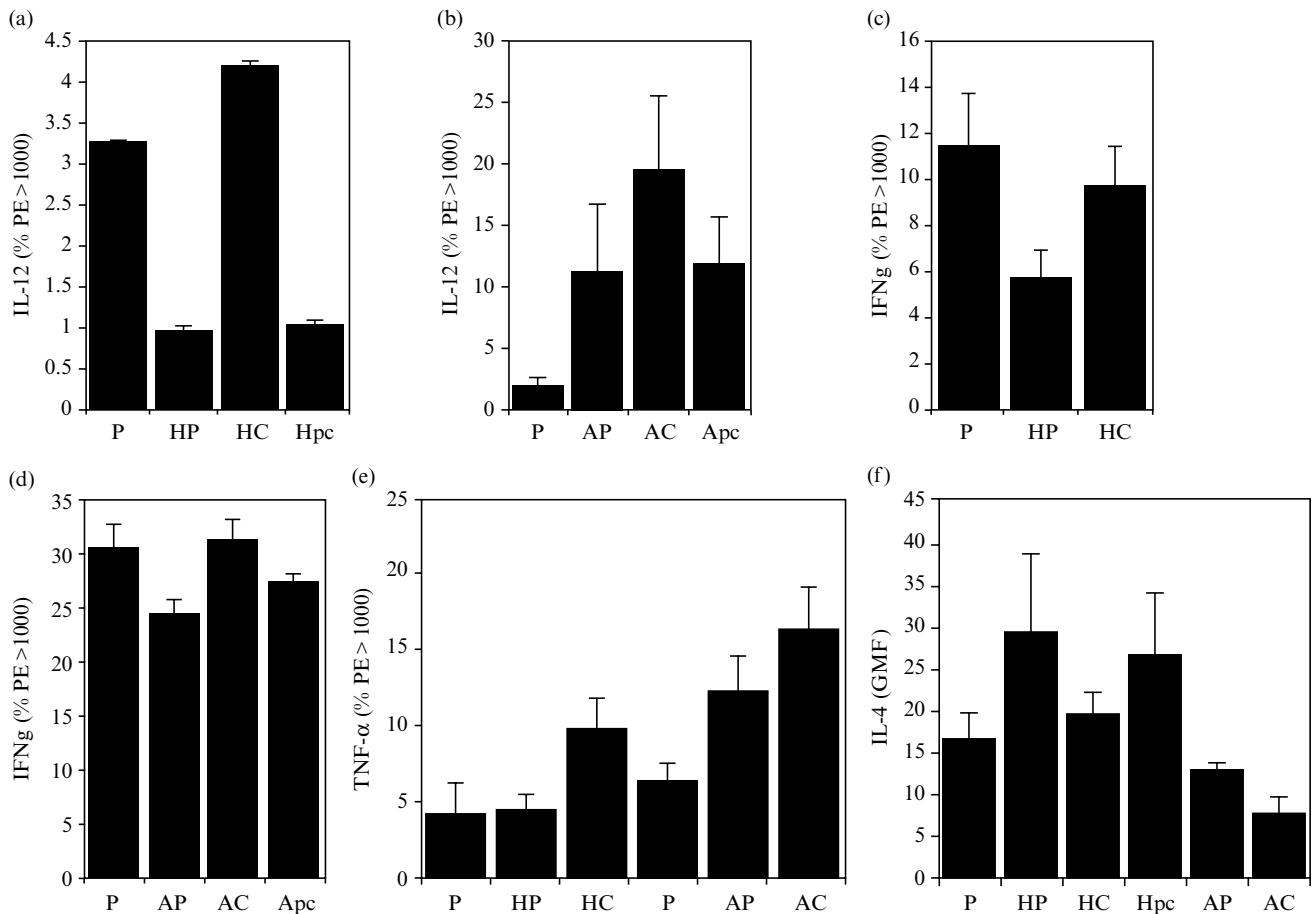


Fig. 3. The effect of CMP treatment with four daily doses of 25 μ g CMP (HC, AC), PBS (HP, AP) or a particulate control (Apc, Hpc), given intranasally on cytokine levels in the spleens of allergen-challenged mice. (a) IL-12 in the Der p model, (b) IL-12 in the Afu model, (c) IFN- γ in the Der p model, (d) IFN- γ in the Afu model, (e) TNF- α in the Der p model and Afu models, (f) IL-4 in the Der p and Afu models. The results are expressed as the percentage of stained cells staining intensely with a PE > 1000, or the geometric mean fluorescence (GMF) after subtraction of background staining. P represents nonsensitized mice treated with PBS.

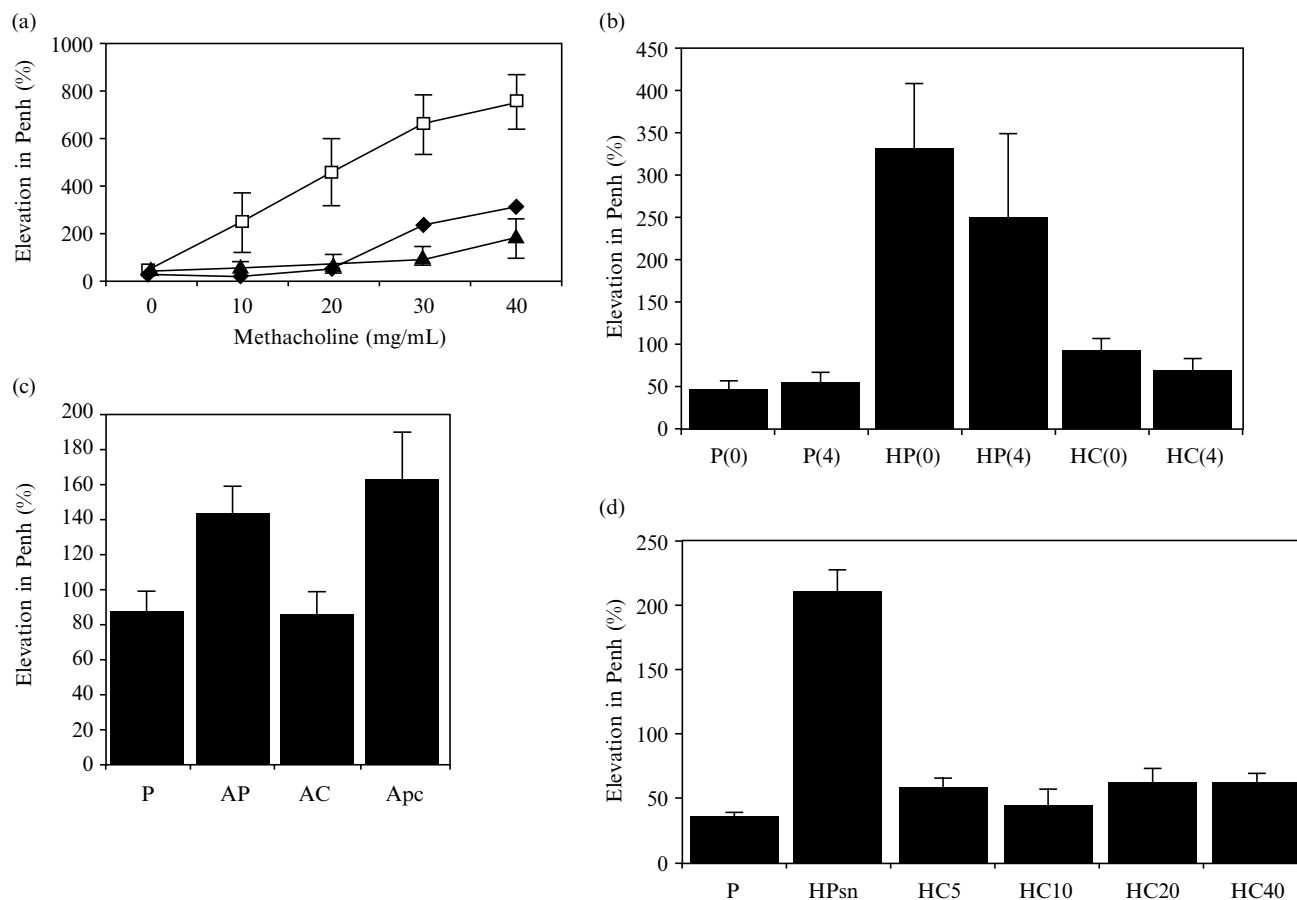


Fig. 4. Airway hyper-responsiveness measured in response to methacholine. (a) Dose-response to methacholine in the Der p model. (b) On the fourth day of treatment [P(0), HP(0), HC(0)] and after re-challenge 4 days after treatment [P(4), HP(4), HC(4)]. (c) Response to methacholine challenge in the Afu model on the fourth day of treatment. Treatment consisted of four daily doses of 25 µg CMP (AC), or PBS (AP) or a particulate control (Apc) given intranasally, to allergen-challenged mice. (d) The effect of four different doses of CMP (5–40 µg) given daily for 4 days to allergen-challenged mice in the Der p allergy model. HPsn is a control treatment with the CMP supernatant after removal of CMP. P represents nonsensitized mice treated with PBS. ♦, PBS; □, Der-PBS; ▲, Der-CMP.

Results are expressed as the elevation in Penh in response to 20 mg/mL of nebulized methacholine. This significant reduction in AHR was also observed in the Afu model on the fourth day of treatment in response to 20 mg/mL methacholine (Fig. 4c, $P < 0.01$). Identical treatment with the particulate control did not reduce airway hyper-responsiveness. In a separate experiment with a new set of Der p-sensitized mice several different doses of CMP were tested (5, 10, 20, 40 µg) using the same allergen challenge and treatment protocol and AHR was measured in response to allergen challenge on day 4, after 3 days of allergen challenge and treatment. All doses produced a significant reduction in AHR (Fig. 4d, $P < 0.005$). To rule out the possibility that a CMP preparation might contain a soluble factor that might account for the observed effects, a control group of mice were treated with the particle-free supernatant from the same CMP preparation (HPsn). No reduction in AHR was observed.

Lung histology

To determine if treatment affected the level of inflammation in allergic mice, lung tissue samples from the Afu study were analysed by a veterinary histopathologist. The peribronchial inflammation of allergen-challenged mice treated with PBS

gave an average score of 2.5 compared with a score of 1.0 for CMP-treated mice also challenged with allergen, representing a 60% reduction in allergen-induced inflammation. Nonsensitized mice treated with PBS gave a score of 0. Lung sections show differences in the degree of inflammation and obstruction of airways after treatment of Afu-sensitized mice with CMP (Figs 5a–c).

Discussion

The objective of the present study was to investigate the effectiveness of the intranasal application of CMP as a treatment for reducing the symptoms of allergy to house dust mite and *A. fumigatus*. Intranasal application was chosen because this might be more relevant for the treatment of allergic symptoms such as allergic rhinitis and asthma in humans by means of a nasal spray or inhalation device. In two different mouse models of allergy, it is demonstrated that intranasal delivery of CMP can significantly reduce serum IgE, Afu-specific IgG1 and peripheral blood eosinophilia and reduce airway hyper-responsiveness. It is of significance that intranasal CMP is able to reduce allergic symptoms even in the presence of

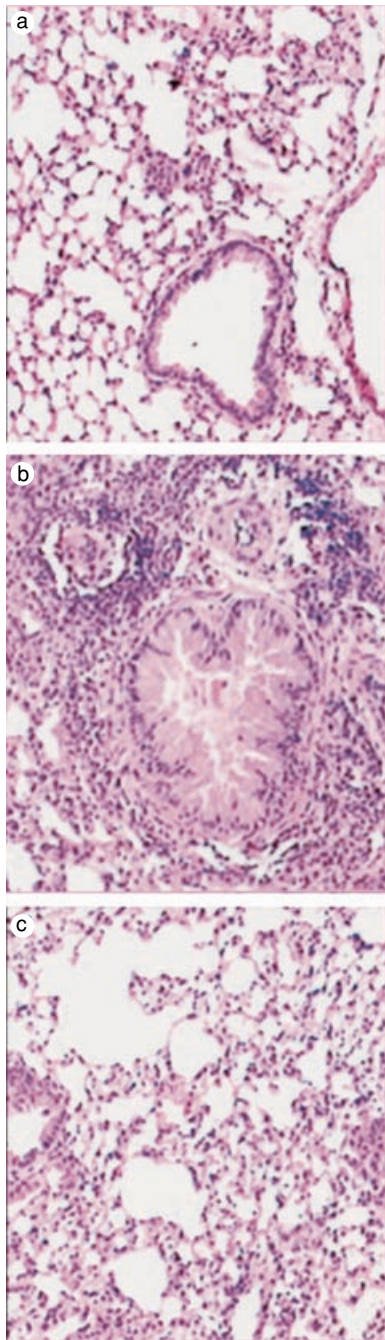


Fig. 5. H&E-stained lung sections of mice from the Afu allergy model after treatment. (a) Normal lung after treatment with PBS, (b) allergic mouse treated with PBS and (c) allergic mouse after intranasal treatment with four daily doses of 25 µg CMP.

sustained allergen challenge as this mirrors the situation encountered by seasonal allergic subjects or those constantly exposed to aeroallergens as in house dust mite allergic asthma. Treatment with CMP would also appear to have a sustained effect on the underlying mechanisms as shown by the significantly reduced IgE, Afu-specific IgG1 and airway hyper-responsiveness after re-challenge with Der p allergen alone the week after treatment. Intranasal CMP treatment elevates IL-12 in the two different models of allergy and would appear to reverse the depression of IL-12 production after allergen

challenge. The higher basal levels of IL-12 in the Afu model is consistent with the fact that Th1-mediated immune responses are elicited by fungal challenge. It is especially interesting that CMP treatment brings about a further increase, which might explain the reduction in airway hyper-responsiveness in this model.

Because IL-12 is produced by activated macrophages and dendritic cells, it is suggested that intranasal treatment with CMP stimulates macrophages in the nasal passages and upper respiratory tract to produce the Th1 cytokine IL-12, which has a major influence in committing Th lymphocytes to a Th1 phenotype and in inhibiting Th2 cell proliferation [11]. This effect is greatly enhanced by the presence of IFN- γ , which is produced by Th1 cells and NK cells in response to IL-12 secretion, and further enhanced by TNF- α produced by macrophages when they interact with CMP. We show that IFN- γ and TNF- α are elevated after treatment even during simultaneous allergen challenge and would appear to reverse the suppression of these important Th1 cytokines. In addition there is a reduction in the level of IL-4 after treatment with CMP, which suggests a modulation in the immune response from Th2 to Th1. Others have demonstrated the importance of IL-12 and IFN- γ in reducing antigen-induced airway hyper-responsiveness [11, 23].

Yoshimi Shibata et al. [28] showed that giving phagocytosable chitin particles orally to mice sensitized to ragweed results in the down-regulation of serum IgE and lung eosinophilia. They also demonstrated, *in vitro*, that chitin significantly reduced the secretion of the Th2 cytokines IL-4, IL-5 and IL-10 and increase the Th1 cytokine IFN- γ produced in spleen cell cultures from ragweed-sensitized mice. The effect was specific to small particulates and could be demonstrated for 1-µm chitin particles or 1-µm polystyrene microspheres coated with *N*-acetyl-D-glucosamine, which is the main component of chitin. No effect was produced by 50-µm chitin particles or soluble chitin and the effect was inhibited by mannan [29] as would be expected if the mannose receptor is involved. In contrast, our findings suggest that it is possible to treat the symptoms of allergy by directly applying chitin microparticles to the respiratory mucosa and affecting a totally different population of immune cells that might be more relevant for treating respiratory allergy. It might be argued that a portion of CMP given intranasally are swallowed by the mice and that the effects that we have found are in part due to oral administration. However, this is unlikely to be the case because our study involved only a few microgram doses given intranasally, whereas Shibata gave doses of 8 mg/day for weeks in order to demonstrate an effect.

It is suggested that the immune system responds to chitin microparticles with a Th1-mediated immune response, which has beneficial effects in reducing the symptoms of allergy. Although the present study involved treating allergic mice, it is interesting to speculate that the application of relatively small amounts of CMP by an appropriate method such as aerosol delivery, might be useful in treating the symptoms of allergic disease in humans, including allergic asthma to house dust mite. It is also possible that macrophages of the nasal mucosa may play a part and that delivery to the alveolar macrophages may not be the only route by which CMP could effect a change. Chitin, derived from crab, lobster or shrimp, has many applications in the medical and pharmaceutical industries and its use as a therapeutic is of particular interest because it is unlikely to have any adverse side-effects, especially when taken nasally in

such small amounts. Chitin is also readily degraded by lysozyme and *N*-acetyl- β -glucosamidase, produced by macrophages, into the simple non-toxic sugar *N*-acetyl- β -glucosamidase [27] and it would be expected that purified chitin, just as chitin found in fungal spores, which are routinely cleared by macrophages, would be readily removed from the system and should present no toxic side-effects.

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