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ORIGINAL PAPER

Treatment with chitin microparticles is protective against lung histopathology in a murine asthma model

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Clinical and Experimental Allergy

Summary

Background Chitin, a natural polysaccharide extracted from shrimp, is a potent T and B cell adjuvant when delivered in the form of chitin microparticles and can shift a polarized T-helper type 2 (Th2) immune response towards a Th1 response.

Objective We investigated the beneficial effects of the intranasal application of chitin microparticles in newborn mice before and after the establishment of a model of allergic asthma.

Methods Mice were grouped as asthma (A), primary prevention (PP), treatment (T), primary prevention+treatment (PPT) and control (C) groups. All mice except controls were sensitized with ovalbumin intraperitoneally and challenged intratracheally to establish the asthma model. Mice in the PP and PPT groups received chitin microparticles intranasally during the newborn period before sensitization. Mice in the PPT and T groups received intranasal chitin microparticles after challenge. Airway histopathology was evaluated in all groups.

Results All of the airway histopathologic parameters of small and medium-sized airways of the T and PPT groups were significantly ameliorated when compared with the asthma model group. In the large airways, thicknesses of basement membrane, epithelium and subepithelial smooth muscle layers of the PPT group and basement membrane thicknesses of the T group were also significantly lower compared with the asthma model group. Comparison of the PP group with the asthma model group revealed significantly reduced goblet cell numbers and significantly reduced epithelial and basement membrane thicknesses in small and medium airways, in addition to significantly reduced basement membrane thicknesses in the medium-sized airways.

Conclusion Intranasal application of microgram quantities of chitin microparticles had a beneficial effect in preventing and treating histopathologic changes in the airways of asthmatic mice.

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Introduction

Although asthma is defined as a reversible diffuse obstructive lung disease, recent studies on the pathogenesis have demonstrated irreversible changes in lung morphology [1, 2]. These structural changes include goblet cell hyperplasia in the epithelium, reticular basement membrane thickening due to deposition of collagen, increased vascularity of the mucosa and thickening of the smooth muscle layer [3].

Clinical symptoms of asthma are related to increases in serum total IgE and allergen-specific IgE due to synthesis of IL-4 and IL-5 cytokines from allergen-specific CD_4^+ T-helper type 2 (Th2) cells [4–6]. Recent studies suggest that shifting the allergen-specific response away from a Th2-polarized response towards a Th1 response may have a therapeutic role in asthma [7, 8]. Various immunomodulators are being evaluated to produce this beneficial shift, including CpG DNA motifs [9–11], bacterial products such as heat-killed *Listeria monocytogenes* [12],

Lactobacillus plantarum [13], BCG [14] and *Mycobacterium vaccae* [15].

Chitin, the second most abundant polysaccharide in nature, is found in fungal cell walls, in the exoskeletons of crustaceans and insects, and in microfilarial sheaths of parasitic nematodes [16–20]. Chitin microparticles (CMP) are non-allergenic, biodegradable and biocompatible particles with sizes in the 1–20 μm range. Chitin already has many applications in the medical, veterinary, cosmetic and environmental industries [21].

Chitin, in the form of phagocytosable microparticles resembles fungal spores and in the same way acts as a potent T and B cell adjuvant [22]. It has also been demonstrated that CMP can shift developing immune responses in a Th1 direction [19, 23]. Mucosal dendritic cells and macrophages are responsible for the phagocytic clearance of microbes and particulates, and by secreting IL-12, IL-18 and TNF-α, promote an effective cell-mediated immune response to inhaled viruses, bacteria, fungal spores and particulates [24, 25]. These cytokines induce IFN-γ production by natural killer 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 IL-4 and IL-5.

Previous studies have demonstrated that oral administration of CMP is effective in down-regulating serum IgE and lung eosinophilia in a mouse model of ragweed allergy [19]. The intranasal application of CMP is an effective treatment for reducing serum IgE and peripheral blood eosinophilia, airway hyper-responsiveness and lung inflammation in allergy models and is accompanied by an up-regulation of IL-12, IFN-γ and TNF-α and a down-regulation of IL-4 production during allergen challenge [23].

In the present study, the protective and therapeutic effect of repeated intranasal CMP administration to neonatal mice on airway histopathology was evaluated in a murine model of asthma.

Methods

Mice

Ten-week-old pregnant BALB/c mice [provided by the Scientific and Technical Research Institute of Turkey (TUBITAK) Gebze, Kocaeli, Turkey] were raised and maintained in a pathogen-free condition with an ovalbumin-free diet. They went into labour on the 21st day of pregnancy and newborn mice were raised and maintained in the same conditions.

The Animal Ethics Committee of Marmara University approved the experimental procedures, and maintenance of animals was in accordance with institutional guidelines.

Chitin microparticles

CMP were obtained as a kind gift from Dr P. Strong of CMP Therapeutics Ltd (Banbury, UK). The chitin was extracted from fresh-water shrimp and milled and sieved through a 50 μm sieve. The particle size ranges from 1 to 20 μm with an average of 4 μm. Endotoxin was measured by *Limulus ameobocyte lysate* (LAL) assay and found to be < 50 endotoxin units/g.

Study groups

Newborn mice were grouped as asthma (A) ($n = 12$), primary prevention (PP) ($n = 7$), treatment (T) ($n = 10$), primary prevention+treatment (PPT) ($n = 13$) and control groups. The control (C) group ($n = 9$) consisted of non-sensitized, non-treated mice.

Establishment of the asthma model

Sensitization and challenge. To establish the asthma model all groups, except the controls were sensitized by intraperitoneal (i.p.) injections of 10 μg of ovalbumin (OVA) (Sigma A-5503, St Louis, MO, USA) in 100 μL of saline administered a total of seven times on each alternate day starting on day 43. Twenty-eight days after the last i.p. injection the mice were challenged with 20 μg of OVA in 10 μL of saline three times 2 days apart by intratracheal (i.t.) instillation as described previously [26, 27].

Intranasal administration of chitin microparticles

The PP group received 100 μg/30 μL of CMP suspension in sterile phosphate-buffered saline (PBS) given intranasally (i.n.) on days 3, 5, 7, 9 and 11 after birth and before sensitization with OVA. Meanwhile, the PPT group received CMP i.n. both on days 3, 5, 7, 9 and 11 and after i.t. challenge with OVA on days 83, 86 and 89. The T group received intranasal CMP only on days 83, 86 and 89 immediately after i.t. challenges with OVA. The study design is summarized in Fig. 1.

Histopathologic analyses

Twenty-four hours after the last i.t. allergen challenge all groups were killed. Lungs were fixed in picric acid and then embedded in paraffin blocks for histopathologic analyses. Paraffin-embedded tissue was sectioned (3–4 μm) and stained with trichrome stain and periodic acid-Schiff (PAS). The histological analyses were carried out with MS Basic Image Analyzer Software (Mikrosistem, Computerized Microscope Systems Co. Ltd, Istanbul, Turkey) adapted to an Olympus BH2-RFCA model microscope (Olympus Optical Co. Ltd, Tokyo, Japan). The airways were

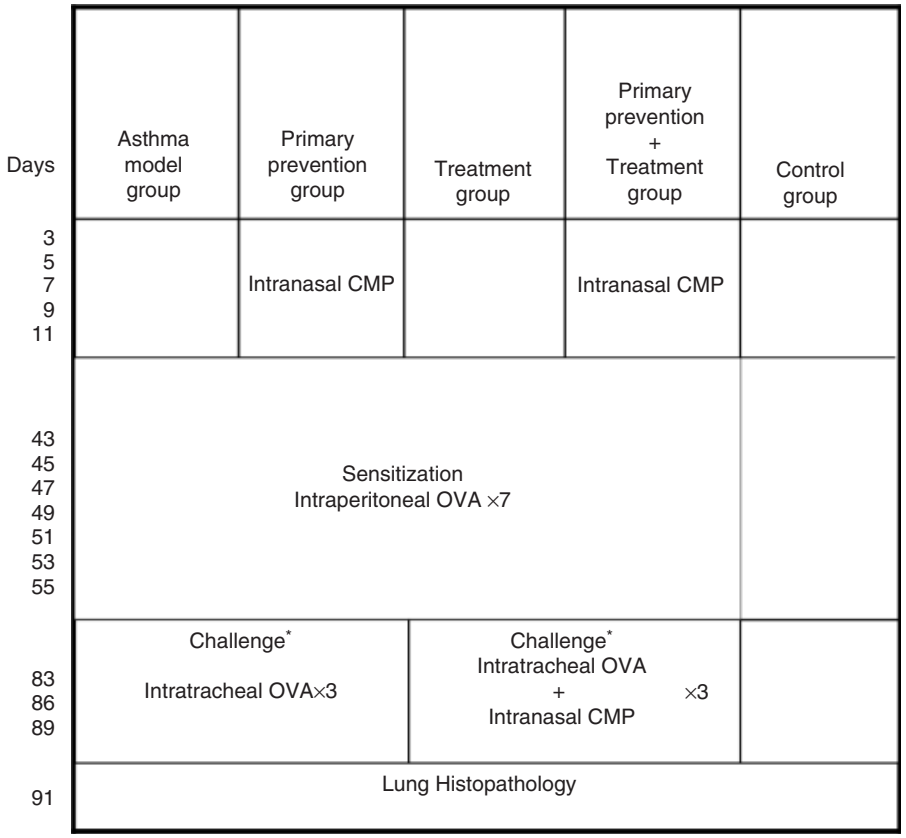

Fig. 1. Study design. *Establishment of asthma model.

classified as small (< 500 μm), medium (500–1000 μm) and large (> 1000 μm) according to their circumferences [15]. Measurements were made on all airways cut in transverse section and free of branching. Measurement of the thicknesses (μm) of epithelial, basement membrane and subepithelial smooth muscle layers and the number of goblet cells on each airway were recorded.

Statistical analyses

Statistical analyses were carried out by the statistical package program of GraphPad Instat version 3.06 (GraphPad Software Inc., San Diego, CA, USA). Dunn's multiple comparisons test was used for comparisons. Difference with a P value of 0.05 or less was considered statistically significant.

Results

When compared with the control group, the asthma model group had significantly higher numbers of goblet cells, increased thicknesses of epithelium, basement membrane and subepithelial smooth muscle layers in small ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively) and medium-sized airways ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively) (Figs 2–5) and a higher number of goblet cells ($P < 0.01$) and increased basement membrane thicknesses ($P < 0.05$) in the large airways. These results revealed that the asthma model was successfully established. Table 1 presents the mean and range values of all histopathologic parameters evaluated.

Primary prevention group

Comparison with the asthma model group. Evaluation of airway histopathology demonstrated that the number of hyperplasic goblet cells in the small and medium-sized airways was significantly lower in the PP group ($P < 0.001$, $P < 0.001$, respectively) and similar significant differences were observed in the thicknesses of basement membrane ($P < 0.01$, $P < 0.001$, respectively) and epithelium ($P < 0.01$, $P < 0.01$, respectively). Also, the subepithelial smooth muscle thicknesses of medium-sized airways in the PP group was significantly lower ($P < 0.001$). No significant difference was observed in those parameters of large airways (Table 1, Figs 2–5).

Comparison with the controls. No significant difference was detected in the number of goblet cells and thicknesses

Table 1. Mean (range) values of evaluated histopathologic parameters of all groups

	PP group (mean (range)), $n = 7$	T group (mean (range)), $n = 10$	PPT group (mean (range)), $n = 13$	A group (mean (range)), $n = 12$	C group (mean (range)), $n = 9$
Goblet cell number (*n*)					
Small	3.0 (0–30)	1.2 (0–17)	0.6 (0–10)	32.9 (0–126)	0 (0–0)
Medium	16.8 (0–93)	13.3 (0–124)	18.1 (0–130)	81.3 (0–333)	0 (0–0)
Large	57.0 (0–114)	32.4 (0–96)	42.5 (0–217)	145.9 (0–361)	0 (0–0)
Epithelial thickness (μm)					
Small	20.3 (14.1–31.9)	19.0 (12.4–29.4)	19.0 (11.3–31.9)	32.9 (20.1–62.0)	16.4 (9.8–28.9)
Medium	23.7 (10.4–55.8)	22.3 (11.3–40.4)	21.4 (13.9–43.5)	31.1 (16.4–61.8)	16.6 (8.7–25.3)
Large	23.3 (19.4–29.4)	22.1 (20.5–24.5)	20.4 (15.7–31.2)	31.5 (15.5–53.3)	20.5 (16.1–32.5)
Basement membrane thickness (μm)					
Small	1.21 (0.7–2.0)	1.12 (0.5–1.5)	1.02 (0.5–1.8)	2.17 (0.9–2.8)	1.06 (0.5–2.6)
Medium	1.29 (0.3–2.6)	1.14 (0.5–1.9)	1.09 (0.5–1.9)	2.00 (1.0–3.9)	1.08 (0.5–1.7)
Large	1.23 (1.0–1.4)	1.12 (0.8–1.5)	1.09 (0.7–1.7)	2.06 (1.2–4.1)	1.14 (0.7–1.6)
Subepithelial smooth muscle thickness (μm)					
Small	3.45 (0–6.5)	2.37 (0–4.1)	2.33 (0–5.7)	5.44 (2.6–9.5)	2.26 (0–5.9)
Medium	3.31 (0–6.2)	2.98 (0–5.0)	2.77 (1.2–5.3)	6.02 (3.1–11.4)	2.87 (0–5.7)
Large	2.33 (0–4.2)	3.38 (2.6–4.5)	3.13 (2.1–6.9)	5.86 (0–12.7)	3.24 (1.8–4.9)

PP, primary prevention; T, treatment; PPT, primary prevention+treatment; A, asthma; C, control; *n*, number.

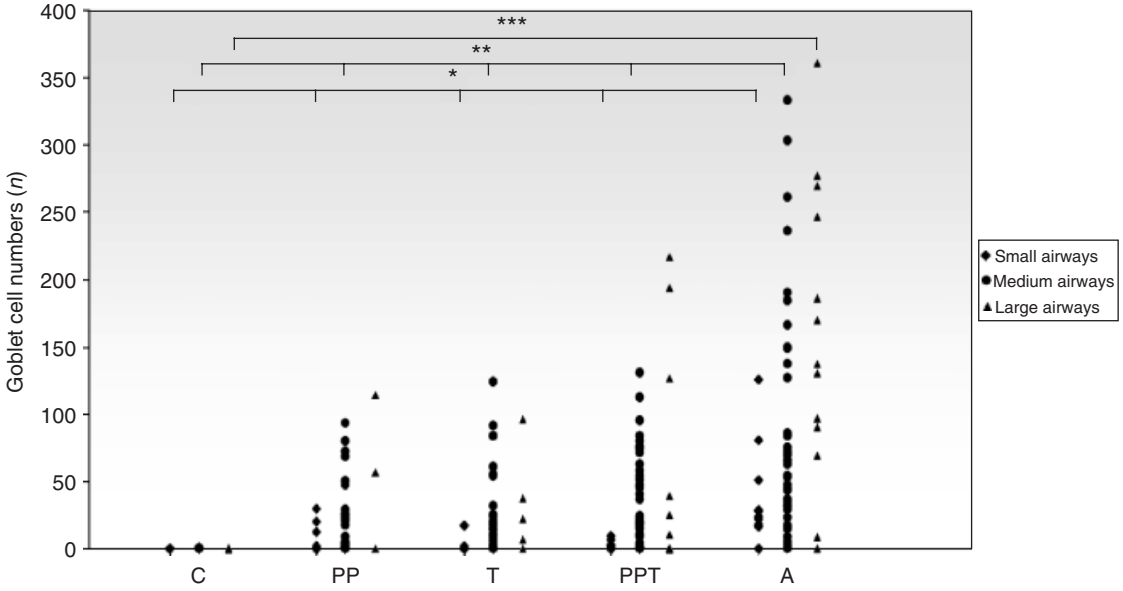

Fig. 2. Goblet cell numbers in all sized airways of all groups. PP, primary prevention group; T, treatment group; PPT, primary prevention+treatment group; A, asthma model group; C, control group. *Small airways: all three treatment groups (PP, T, PPT) and controls revealed statistically significantly lower goblet cell numbers compared with the asthma model group. There was no significant difference between treatment groups and controls. Furthermore, comparison between treatment groups revealed no significant difference. A vs. PP ($P < 0.001$), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS). **Medium airways: comparison of all three treatment groups with the asthma model group revealed significantly lower goblet cell number, whereas comparison with controls revealed significantly higher numbers. On the other hand, there was no significant difference in between treatment group comparisons. A vs. PP ($P < 0.001$), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C ($P < 0.01$), T vs. C ($P < 0.05$), PPT vs. C ($P < 0.01$), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS). ***Large airways: there was no statistically significant difference in comparison of the treatment groups with asthma model and control groups. Also, between treatment groups comparisons revealed no significant difference. Only the asthma model group had significantly higher goblet cell numbers than controls. A vs. PP (NS), A vs. T (NS), A vs. PPT (NS), A vs. C ($P < 0.01$), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS), Dunn's multiple comparisons.

of the basement membrane and epithelium of small airways, or the thicknesses of basement membrane and subepithelial smooth muscle layer of medium-sized airways, and in all of the parameters of large airways (Table 1, Figs 2–5).

Primary prevention+treatment group

Comparison with the asthma model group. Mice in the PPT group had significantly lower goblet cell numbers in small and medium-sized airways ($P < 0.001$, $P < 0.001$, respectively). Also there was significantly less thickening of basement membrane ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively), epithelium ($P < 0.001$, $P < 0.001$, $P < 0.05$, respectively) and subepithelial smooth muscle layers ($P < 0.001$, $P < 0.001$, $P < 0.01$, respectively) in all three sized airways (Table 1, Figs 2–5).

Comparison with the controls. Goblet cell numbers, thicknesses of basement membrane, epithelium and subepithelial smooth muscle layers of small and large airways as well as thicknesses of basement membrane and subepithelial smooth muscle layers of medium-sized airways were found to be not statistically different from controls (Table 1, Figs 2–5).

Treatment group

Comparison with the asthma model group. When compared with the asthma group all histopathologic parameters of small ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively) and medium-sized airways ($P < 0.001$ $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively) as well as the thicknesses of the basement membrane of large airways ($P < 0.05$) were found to be statistically significantly lower in the T group (Table 1, Figs 2–5).

Comparison with the controls. All of the evaluated histopathologic parameters were found not to be significantly different from the controls except for the goblet cell numbers and epithelial thicknesses in medium-sized airways ($P < 0.05$, $P < 0.001$, respectively) (Table 1, Figs 2–5).

Comparison of primary prevention, treatment group and primary prevention+treatment group groups

In general we demonstrated ameliorating effects of intranasal CMP administration in all of the treatment groups (PP, T, PPT) on the airway histopathologic features in the murine model of allergic asthma. No statistically

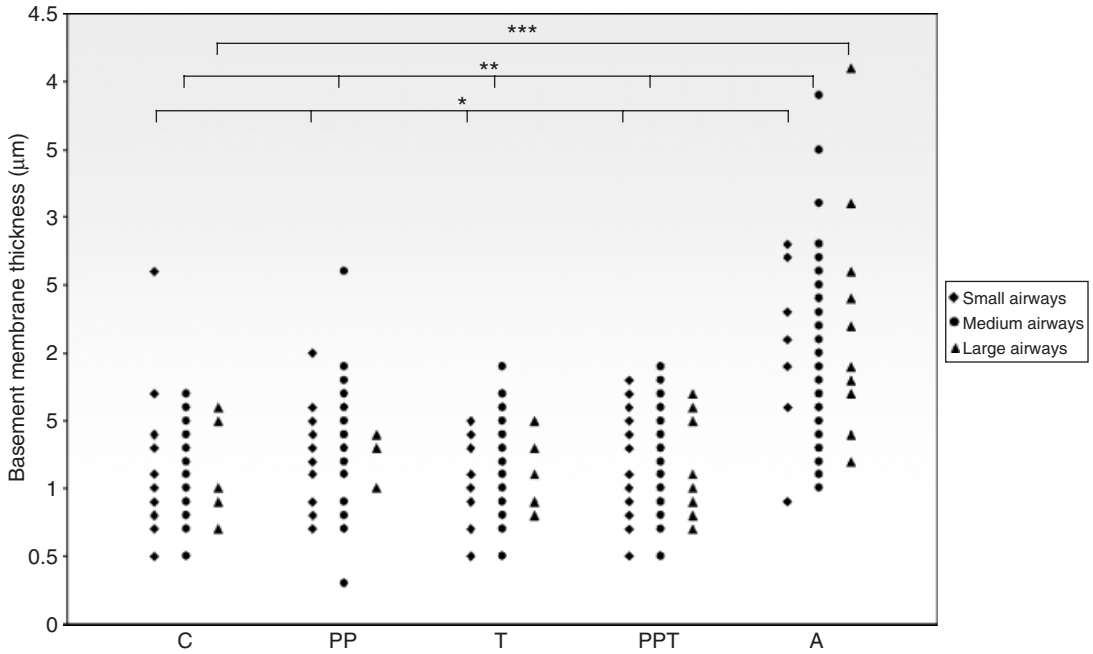

Fig. 3. Basement membrane thicknesses in all sized airways of all groups. PP, primary prevention group; T, treatment group; PPT, primary prevention+treatment group; A, asthma group; C, control group. All three sized airways of treatment groups and controls revealed significantly decreased basement membrane thicknesses when compared with the asthma model group, except for the large airways of the PP group. Comparison of treatment groups with each other and controls revealed no statistically significant difference. *Small airways: A vs. PP ($P < 0.01$), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS). **Medium airways: A vs. PP ($P < 0.001$), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS). ***Large airways: A vs. PP (NS), A vs. T ($P < 0.05$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.05$), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS), Dunn's multiple comparisons.

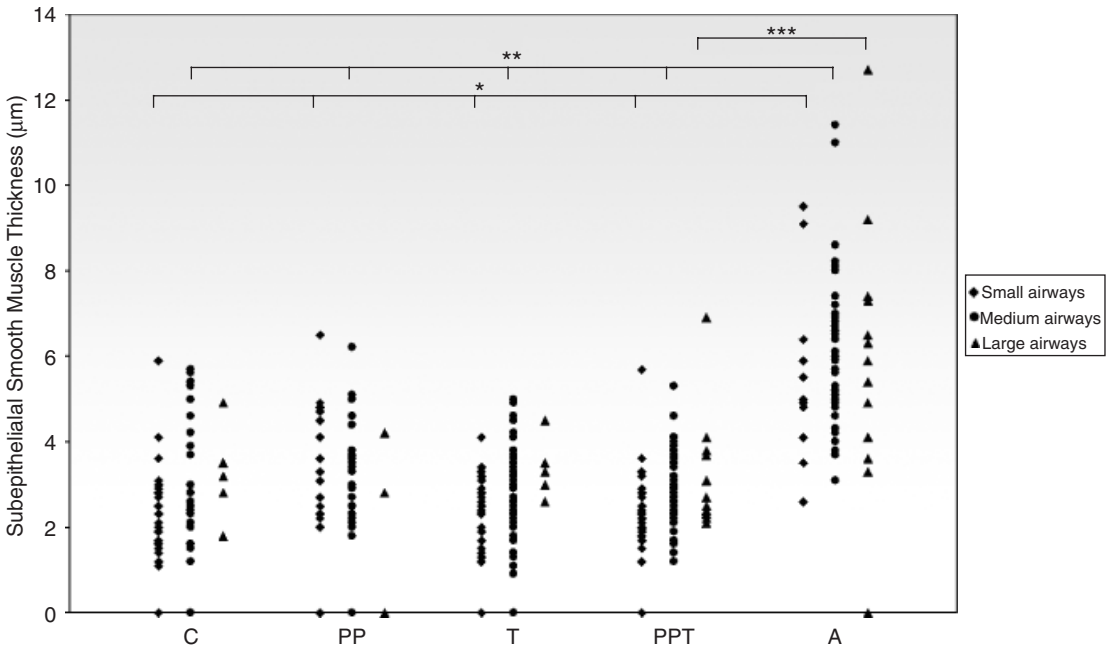

Fig. 4. Subepithelial smooth muscle layer thicknesses in all sized airways of all groups. PP, primary prevention group; T, treatment group; PPT, primary prevention+treatment group; A, asthma group; C, control group. *Small airways: T, PPT and control groups had significantly lower subepithelial smooth muscle thicknesses compared with the asthma model group. Moreover, T and PPT groups were not statistically different from controls. In between-groups comparisons, the PPT group had statistically significantly lower subepithelial smooth muscle thicknesses compared with the PP group. A vs. PP (NS), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C ($P < 0.05$), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT ($P < 0.05$), T vs. PPT (NS). **Medium airways: comparisons of all treatment groups and controls revealed statistically significantly lower subepithelial smooth muscle thicknesses than the asthma model group. There was no significant difference between treatment groups and controls. Furthermore, comparison between treatment groups revealed no significant difference. A vs. PP ($P < 0.001$), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS). ***Large airways: among the treatment groups, only the PPT group had significantly lower subepithelial smooth muscle thicknesses than the asthma model group. Compared with controls none of the treatment groups revealed significant differences. There was no difference in between treatment groups. A vs. PP (NS), A vs. T (NS), A vs. PPT ($P < 0.01$), A vs. C (NS), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS), Dunn's multiple comparisons.

significant differences were observed in the comparison of the PP group with the T group in all evaluated parameters. No difference was seen in the comparison of the PPT group with the T group. On the other hand, the PPT group was found to have significantly lower subepithelial smooth muscle thicknesses of small airways compared with the PP group ($P < 0.05$). Furthermore, the PPT group seemed to ameliorate basement membrane, epithelial and subepithelial smooth muscle thicknesses in the large airways when compared with the asthma model group. Taken together, these results support the effectiveness of intranasal CMP given both before sensitization and during allergen challenge (Fig 6).

Discussion

In this study the beneficial effects of intranasal CMP administration on lung histopathology in a murine model of allergic asthma were demonstrated in:

a. the neonatal period, before sensitization with OVA,
b. during i.t. OVA challenge,
c. both before sensitization and during OVA challenge.

Strong et al. [23] studied the effectiveness of CMP when given i.n. as a treatment for the symptoms of allergic asthma and demonstrated effectiveness in two different mouse models of allergy, namely to allergen extracts of *Dermatophagoides pteronyssinus* (Der p) and *Aspergillus fumigatus* (Afu). They reported that intranasal application of microgram quantities of CMP given 1 or 2 h after an intranasal allergen challenge is an effective treatment for reducing serum IgE and peripheral blood eosinophilia. Treatment also reduced airway hyper-responsiveness, which is one of the characteristic features of asthma, in both allergy models. Furthermore, 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 staining. The Th1 cytokine IL-12 was significantly elevated fourfold in the Der p model, and 1.7-fold in the Afu model, after intranasal treatment. Moreover, IFN-γ was elevated 1.7-fold in the Der p model and 1.3-fold in the Afu model. Comparison of the geometric mean fluorescence of spleen cells stained for the Th2 cytokine IL-4

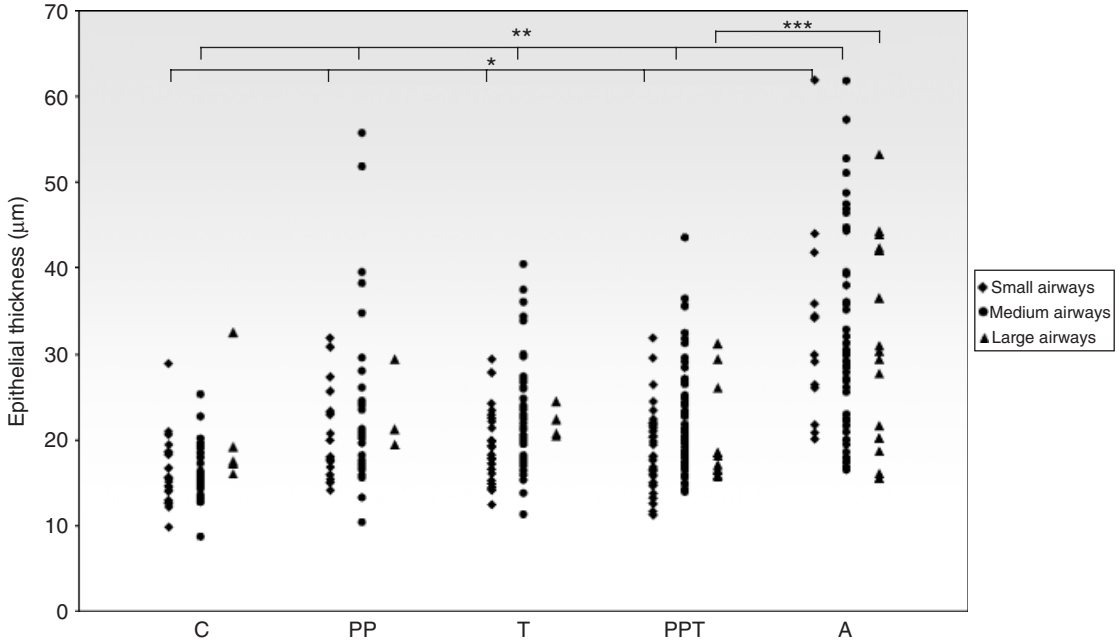

Fig. 5. Epithelial thicknesses in all size airways of all groups. PP, primary prevention group; T, treatment group; PPT, primary prevention+treatment group; A, asthma group; C, control group. *Small airways: all treatment groups and controls had significantly lower epithelial thicknesses than the asthma model group. In comparison with controls, treatment groups demonstrated no significant difference. Comparison between treatment groups revealed no significant difference. A vs. PP ($P < 0.01$), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS). **Medium airways: all treatment groups and controls had significantly lower epithelial thicknesses than the asthma model group. On the other hand, all treatment groups had a statistically more thickened epithelial layer than controls. There was no significant difference in between treatment group comparisons. A vs. PP ($P < 0.01$), A vs. T ($P < 0.001$), A vs. PPT ($P < 0.001$), A vs. C ($P < 0.001$), PP vs. C ($P < 0.001$), T vs. C ($P < 0.001$), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS). ***Large airways: Among treatment groups only the PPT group had statistically significantly lower epithelial thicknesses compared with the asthma model group. There was no significant difference in comparisons of treatment groups with controls, as well as in between-group comparisons. A vs. PP (NS), A vs. T (NS), A vs. PPT ($P < 0.05$), A vs. C (NS), PP vs. C (NS), T vs. C (NS), PPT vs. C (NS), PP vs. T (NS), PP vs. PPT (NS), T vs. PPT (NS), Dunn's multiple comparisons.

showed a decrease of 34% in the Der p model and 27% in the Afu model after intranasal treatment with CMP [23].

Also, Shibata et al. [19] demonstrated that oral administration of milligram quantities of CMP is effective in down-regulating serum IgE and lung eosinophilia in a mouse model of ragweed allergy when given during the period of sensitization and allergen challenge. Furthermore, to elucidate the inhibitory mechanisms of Th2 responses, spleen cells isolated from the ragweed-immunized mice were cultured in the presence of ragweed and/or CMP for 3 days. Ragweed alone stimulated the production of IL-4, IL-5 and IL-10, but not IFN-γ. Ragweed/chitin stimulation resulted in significant decreases of IL-4, IL-5 and IL-10 levels and the production of IFN-γ. Moreover, spleen cells isolated from the chitin-treated mice showed ragweed-stimulated IFN-γ production and significantly lower levels of the Th2 cytokines, suggesting that the immune responses were redirected towards a Th1 response [19].

Results of the current study are in accordance with the findings of previous studies [19–23] demonstrating that CMP given i.n. before sensitization and during allergen challenge ameliorates histopathologic changes in the goblet cell numbers, basement membrane, epithelial and subepithelial smooth muscle layer thicknesses of asthmatic airways in mice.

To date, there have been no data on the effectiveness of CMP administered i.n. in the newborn period as a possible prophylactic treatment to prevent the development of asthma in mice. One of the most significant findings of this study is that the intranasal application of microgram quantities of CMP during the neonatal period would seem to have a protective effect against the development of the asthma model in mice. Our results revealed that in the airways of mice treated prophylactically with CMP, the goblet cell numbers, and thicknesses of epithelium, basement membrane and subepithelial smooth muscle layers were all significantly lower and reduced to levels not significantly different from non-sensitized control mice. Moreover, the results of this study supported the idea that the most effective strategy would be a combination of preventive and therapeutic approaches (PPT group) using CMP given i.n..

On the other hand, we acknowledge the lack of experiments in our study on airway hyper-responsiveness and inflammation as well as on profiles of Th1 and Th2 cytokines at protein or mRNA levels with or without chitin treatment. Furthermore, the present study had not shown

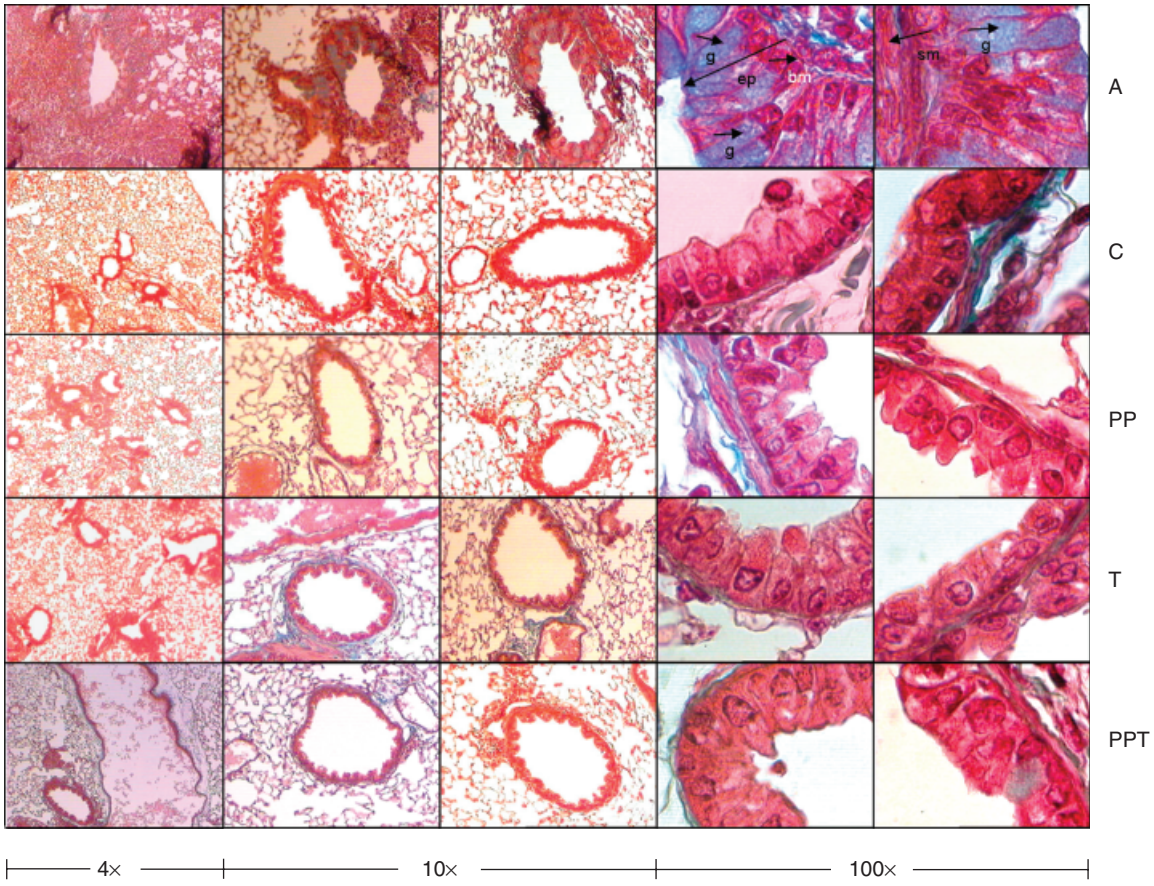

Fig. 6. Histological views of airways. In the high-power (× 100) trichrome-stained examinations of airway sections of mice in the asthma model group (A), numerous goblet cells (g), increased epithelial (ep), basement membrane (bm) and subepithelial smooth muscle layer (sm) thicknesses were seen. These evaluated parameters appeared normal in sections from control (C) group mice. The appearance of airways of mice from all primary prevention (PP), treatment (T) and primary prevention+treatment (PPT) groups were similar to the control group.

what the long-term effect of treatment with CMP upon repeated allergen challenges would be in those mice. To answer that, it would be important to continue allergen challenges over a longer period to see if the protective effect conferred by CMP would remain.

It was assumed that the anti-allergic and anti-asthma effect of CMP was due at least in part to the local modulation of the cytokine environment in the airways as suggested by Strong et al. [23] That effect presumably began with the uptake of CMP by macrophages and other phagocytic cells in the respiratory mucosa. Shibata et al. [19] demonstrated that chitin in the form of microparticles ($< 10\,\mu m$) stimulated production of IL-12, TNF-α and IFN-γ *in vitro* from mouse spleen cell cultures and this effect was inhibited by soluble chitin or mannan confirming the importance of phagocytic uptake and likely involvement of the macrophage mannose receptor [28].

Our results point to the protective effect of intranasal chitin microparticles given in the newborn period against the establishment of asthma. Considering the fact that modern therapeutic modalities have limited efficacy in reversing already established asthmatic changes in the airways, interventions before the sensitization period would be most beneficial in terms of prevention. However, before the suggestion of chitin as a safe prophylactic strategy in the prevention of asthma in human, biological parameters such as airway hyper-reactivity, inflammation and cytokine responses have to be delineated.

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