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Oxidized Cellulose Binding to Allergens with a Carbohydrate-Binding Module Attenuates Allergic Reactions

Nir Shani,* Ziv Shani,[†] Oded Shoseyov,* Rufayda Mruwat,[‡] and David Shoseyov[§]

Grass and mite allergens are of the main causes of allergy and asthma. A carbohydrate-binding module (CBM) represents a common motif to groups I (β -expansin) and II/III (expansin-like) grass allergens and is suggested to mediate allergen-IgE binding. House dust mite group II allergen (Der p 2 and Der f 2) structures bear strong similarity to expansin's CBM, suggesting their ability to bind carbohydrates. Thus, this study proposes the design of a carbohydrate-based treatment in which allergen binding to carbohydrate particles will promote allergen airway clearance and prevent allergic reactions. The aim of the study was to identify a polysaccharide with high allergen-binding capacities and to explore its ability to prevent allergy. Oxidized cellulose (OC) demonstrated allergen-binding capacities toward grass and mite allergens that surpassed those of any other polysaccharide examined in this study. Furthermore, inhalant preparations of OC microparticles attenuated allergic lung inflammation in rye grass-sensitized Brown Norway rats and OVA-sensitized BALB/c mice. Fluorescently labeled OC efficiently cleared from the mouse airways and body organs. Moreover, long-term administration of OC inhalant to Wistar rats did not result in toxicity. In conclusion, many allergens, such as grass and dust mite, contain a common CBM motif. OC demonstrates a strong and relatively specific allergen-binding capacity to CBM-containing allergens. OC's ability to attenuate allergic inflammation, together with its documented safety record, forms a firm basis for its application as an alternative treatment for prevention and relief of allergy and asthma. *The Journal of Immunology*, 2011, 186: 000–000.

Asthma prevalence in recent decades is increasing, and is estimated to affect as many as 300 million individuals worldwide (1, 2). Approximately 40% of the occurrences of asthma are attributed to allergies (3, 4). The increase in asthma prevalence has been correlated with a rise in other allergic disorders, such as eczema and rhinitis (5).

Grass pollen allergens constitute one of the main triggers of allergy, affecting as many as 20% of the general population (6, 7). Group I allergens, the most potent grass pollen allergens found in most grasses and corn species, are termed β -expansin and contain a C-terminal carbohydrate-binding module (CBM) (8, 9). A three-dimensional model of the CBM of the rye grass pollen allergen Lol1 pI (β -expansin), which demonstrates resemblance to that of the bacterial family-III CBM, bears allergenic properties that are associated with its CBM structure (10). Similarly, expansin-like proteins of major grass allergen groups II and III bear homology to the CBM portion of β -expansin (9, 10). Other potent allergens (mite, olive, latex, and fruits) also contain a CBM, suggesting it has a critical role in prompting allergic responses (10–14).

The realization that a large proportion of grass and other allergies is provoked by CBM-containing proteins that possess an inherent affinity to polysaccharides has prompted the development of an inhalant polysaccharide-based compound designed to inhibit the onset of allergic reactions through capture and clearance of CBM-containing allergens prior to their encounter with dendritic cells and IgE (see Fig. 1A, 1B). An effective compound must demonstrate high allergen-binding capacity, delayed solubility to allow for thorough clearance by the mucociliary system, biocompatibility, and bioabsorbability. The aim of this work was to identify an allergen-binding polysaccharide and to examine its ability to prevent allergy and asthma.

Materials and Methods

OC preparation

OC was prepared from either 20 μ m Avicel (microcrystalline cellulose; FMC BioPolymer, Philadelphia, PA) or Tencel (Lenzing Fibers, Grimsby, U.K.). Preparation and carboxyl content determinations were performed according to the protocol described by Kumar and Yang (15). All oxidized cellulose (OC) types were micronized by vortex milling before use (Super Fine, Yokneam, Israel) to a size of $\sim$ 10 μ m (see Supplemental Fig. 1).

Pollen protein extraction

Lolium perenne pollen (Allergon AB, Ängelholm, Sweden) or native *Lolium rigidum* pollen from wild plants was suspended in endotoxin-free PBS (100 mg/ml), incubated at room temperature for 20 min, and then centrifuged. The supernatants were filtered through a 0.45- μ m polyvinylidene difluoride filter (Millipore, Bedford, MA).

Allergen-binding assay

SDS-PAGE-based evaluation. Soluble pollen proteins or standardized mite *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* allergenic mixed extract (ALK, Hørsholm, Denmark) were incubated (1 h, room temperature) with 20 mg microparticles of either OC prepared from Avicel, or with unmodified Avicel. Following centrifugation (1 min, 10,000 $\times$ g, room temperature), the supernatant (unbound fraction) was collected, whereas the pellet (bound fraction) was washed with PBS before collection. Fractions were separated by SDS-PAGE and stained with either Coomassie blue or silver stains.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BN, Brown Norway; CBM, carbohydrate-binding module; IR, infrared; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; OC, oxidized cellulose; RMSD, root mean square deviation; SSM, secondary structure matching.

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Bradford-based evaluation. Soluble *L. rigidum* or *L. perenne* pollen proteins were incubated with OC prepared according to Kumar and Yang (15) or commercial OC (OKCEL H-L or Ca-L; Synthesia, Pardubice-Semtin, Czech Republic), Avicel, micro granular carboxymethyl cellulose CMC (Whatman, Maidstone, U.K.), and Chitosan (Sigma-Aldrich, St. Louis, MO) in saline. The unbound protein content was calculated using the standard Bradford protein assay, and the amount of OC-bound protein was extrapolated.

Induction of allergic inflammation

Animals. BALB/c mice and Brown Norway (BN) rats (Harlan Laboratories) were maintained under conventional conditions and used according to protocols approved by the Hebrew University Ethics Committee on Research Animal Care.

Experiment design

BN model. BN male rats (50–70 g) were sensitized on day 0 with a s.c. injection of 1 mg *L. perenne* pollen protein plus 200 mg aluminum hydroxide/1 ml PBS and an i.p. injection of 0.1 ml *Bordetella pertussis* (6×10^9 , after heat inactivation; Pasteur Marieux, France). On day 13, rats either untreated (group 1) or pretreated with OC powder inhalant for 5 min (0.45 mg/l; details in Supplemental Fig. 2) (group 2) were challenged with a 20-min exposure to a 1 mg/ml *L. perenne* pollen inhalant protein preparation. Pollen inhalation was performed with an ultrasonic nebulizer (LS 230 System Villeneuve Sur Lot, France).

BALB/c model. Female BALB/c mice (7–8 wk old) were sensitized on days 0 and 14 with 20 µg by i.p. injection of OVA (grade V; Sigma-Aldrich) mixed with 2 mg aluminum hydroxide (Merck, Darmstadt, Germany). On days 21 and 22, group 1 was challenged with a 50 mg/ml OVA inhalant preparation for 20 min, whereas group 2 was exposed for 4 min to an OC powder inhalant (800 mg, continuously dispersed by a bulb in a 2-L container), immediately followed by an OVA allergen challenge. Group 3 was treated with PBS inhalation only. Group 4 naive mice were not sensitized to OVA and left untreated.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed on day 15 (BN rat model) or day 23 (BALB/c mouse model) after sacrificing by exsanguination through the abdominal aorta. BAL was performed with 3×5 ml (BN model) or 3×1 ml (BALB/c model) cold PBS. Total cell count was recorded, and a differential count of 250 cells was performed, using standard morphological criteria after fixation with cytospin and staining with May-Grunwald Giemsa (Riedel de Haen, Seelze, Germany). Mouse IL-5 and IL-4 levels were measured in the BALB/c first 1-ml BAL fraction using commercially available detection kits (BD Biosciences, Franklin Lakes, NJ).

Determination of anti-OVA-specific Abs in the serum

OVA-specific IgE and IgG sera Ab titers were determined by ELISA. Briefly, 96-well microtiter plates were coated with 5 µg/ml OVA diluted in NaHCO₃ buffer (pH 9.5). After overnight incubation at 4°C, plates were washed and blocked with 2% BSA. Serial dilution of sera was added to each well for 1 h at room temperature. Plates were washed and incubated with anti-mouse IgE or IgG conjugated to HRP (Bethyl Laboratories, Montgomery, TX) diluted in 1% BSA-PBS buffer for 1 h at room temperature. After extensive washing, wells were developed by the 1-Step Ultra tetramethylbenzidine substrate (Pierce Biotechnology, Rockford, IL), and absorbance at 652 nm was determined using a microplate reader.

Lung pathology

Lungs were fixed by inflation (pressure 20 cm H₂O) with 4% formaldehyde and embedded in paraffin. Histological sections were randomly selected from the middle third and were stained with H&E for assessment of interstitial and peribronchial inflammation. Assessment of peribronchial inflammation was performed for each bronchi by an investigator (D.S.) who was blinded to the treatment groups, as previously described (16). Pathology scores were defined as follows: 1, normal; 2, mild; 3, moderate; and 4, marked. Microscopic images were captured using an IX51 Olympus microscope equipped with a DP71 Olympus camera ($\times 20$ lens). Images were analyzed with CellA software version 2.8.

Splenocyte proliferation

Single-cell suspensions in RPMI 1640 (Biological Industries, Beit Haemek, Israel) were prepared from spleens of OVA-sensitized mice, and erythrocytes were depleted. After three washes, the cells were resuspended in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Biological Industries); plated at 5×10^5 spleen cells/well

in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) $\pm$ 0.5 mg/ml OVA; and cultured for 48 h. Cell proliferation was examined using WST-1 cell proliferation reagent (Roche Diagnostics, Mannheim, Germany).

Preparation of OC-Cy5.5 conjugates

OC (100 mg), 125 µl 100 mg/ml *N*-(3-dimensionalimethylaminopropyl)-*N'*-ethylcarbodiimide (Sigma-Aldrich), and 50 µl 2.2 mM Cy5.5 hydrazide (GE Healthcare, Piscataway, NJ) were mixed in 10 ml absolute ethanol and incubated for 1 h in the dark. The Cy5.5-OC conjugate was then consecutively washed in 100, 90, 50% absolute ethanol and in double distilled water to remove unbound dye. The conjugates were then washed again with absolute ethanol and left to dry overnight at room temperature.

In vivo imaging of Cy5.5-OC

Cy5.5-OC (50 µl 10 mg/ml in PBS) was administered intranasally to 20 (in vivo imaging)- or 8 (organ distribution)-wk-old, anesthetized BALB/c female mice. Near-infrared (IR) images were acquired at the indicated time points using the IVIS 100 (Xenogen) using excitation = Cy5.5, emission = Cy5.5 bandpass filters.

Long-term exposure to OC inhalant

Male and female Wistar rats (180–200 g) were subjected to either 0.45 mg/l OC particle powder inhalation three times per week for 15 min at each exposure session or left untreated (control) for a period of 21 d. Rat weights were periodically recorded. Rats were sacrificed on day 21, followed by BAL, performance of a differential cell count, and lung fixation and staining for pathologic evaluation, as described for the BN rat model.

Statistical analysis

Results were compared between groups with paired Student *t* test using JMP statistical software (SAS Institute, Cary, NC). Pathology scores were defined as ordinal nonparametric values, and the differences between groups were analyzed either by the Mann-Whitney rank sum test (BN model) or by the Kruskal-Wallis test (BALB/c model) using statistical software (GB-STAT; Dynamic Microsystem, Silver Spring, MD). A *p* value < 0.05 was considered statistically significant.

Results

Binding capacity of OC to grass and mite allergens

The first stage of our research involved a broad search for a polysaccharide-bearing binding capacities toward rye grass allergens. Carob galactomannan, pullulan, guar galactomannan, pectic galactan, oat β -glucan, barley β -glucan, 1,4- β -D-mannan, and tamarind xyloglucan polysaccharides failed to demonstrate substantial allergen-binding capacities (data not shown) (17). Similarly, unmodified crystalline cellulose demonstrated very poor binding capacity to the tested allergens (see Fig. 2A*I*). In contrast, OC, a biodegradable derivative of cellulose produced from crystalline cellulose, exhibited significant binding capacity to grass pollen allergens, in general, and potent binding capacity to expansin-like proteins (group II/III grass allergens), in particular (expansin-like proteins, as was confirmed by liquid chromatography-tandem mass spectrometry [LC-MS/MS], are marked in a red circle), are enriched in the bound fraction (Fig. 2A*I*). As it is demonstrated in Fig. 2A*II*, the CBM structures of β -expansin and expansin-like grass allergens share strong structural homology to group II major mite allergens Der p 2 and Der f 2, indicating the ability of mite allergens to bind polysaccharides. Thus, we assessed the affinity of OC to mite allergens, which are potent elicitors of allergy (18). As in the case of grass expansin-like allergens, OC exhibited a significant binding capacity toward mite allergens, in general, and marked affinity toward Der f 2 and Der p 2 (Fig. 2A*III*; compare the bound Der p 1 with the bound Der p 2, both confirmed by LC-MS/MS). As illustrated with grass allergens (see Fig. 2A*I*), OC-binding capacity to mite allergens was far superior to that of crystalline cellulose (see Fig. 2A*III*).

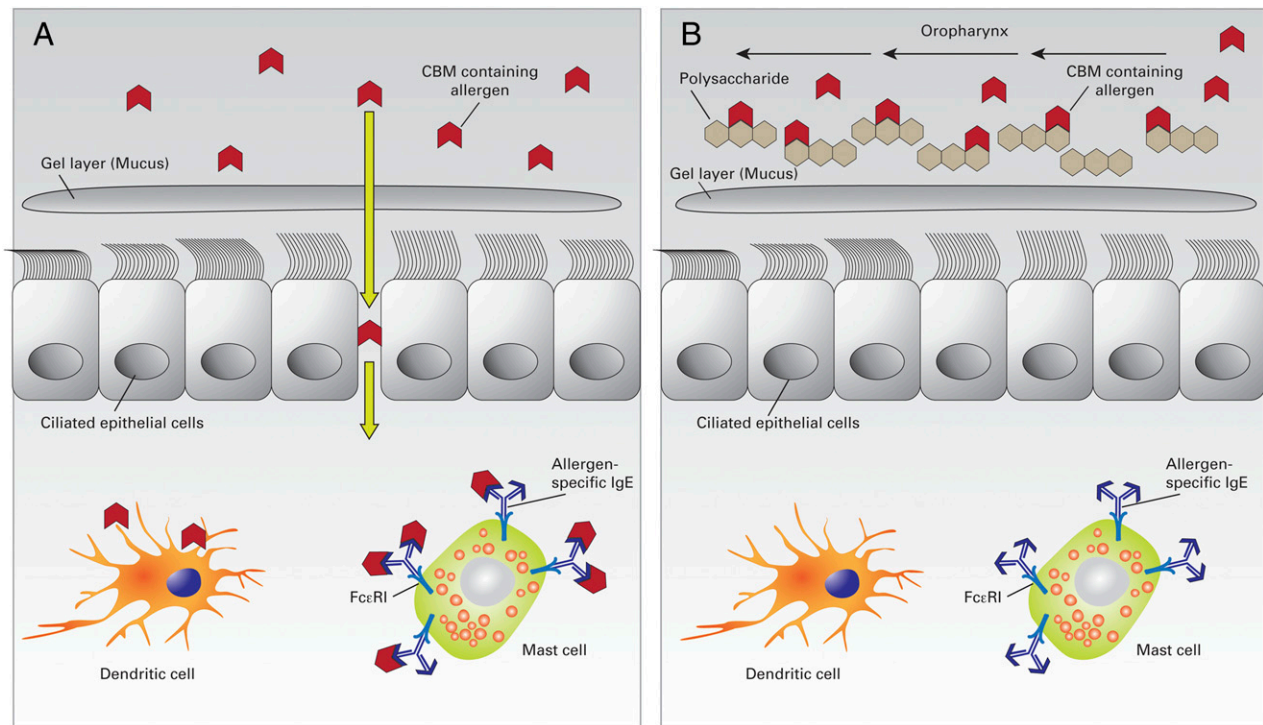


FIGURE 1. A theoretical model describing allergy prevention by polysaccharide microparticles. *A*, CBM-containing aeroallergens inhaled into the airway penetrate the epithelial cell layer using its intrinsic penetration abilities. In atopic individuals, CBM-containing allergens reach the submucosal layer, access dendritic cells, or attach to IgE-bearing mast cells and initiate allergic reactions. *B*, Pretreatment with a polysaccharide microparticle inhalant blocks CBM-containing allergens through specific CBM-based allergen–polysaccharide interactions. Allergen–polysaccharide complexes lose their ability to penetrate the epithelial barrier and are efficiently cleared from the airways by the mucociliary clearance mechanism.

Thus, OC demonstrated potent binding of grass and mite allergens, in general, and to the CBM-containing allergens, in particular.

The contribution of OC oxidation levels to its allergen-binding capacity

OC is a cellulosic polymer in which the hydroxyl group at carbon 6 of the glucose monomer is oxidized to a carboxylic group. In the course of this gradual oxidation reaction, copolymers consisting of varying ratios of glucose and glucuronic acid units are created. To this end, the pollen allergen-binding capacity of batches of OC prepared to contain increasing quantities of carboxylic groups was assessed (all the OC batches were prepared from microcrystalline cellulose). A significant increase in allergen-binding capacity was observed with elevated (~9–16% w/w) oxidation levels (see Fig. 2*BI*). OC allergen-binding capacities plateaued at oxidation levels exceeding 16% w/w. Thus, OC allergen-binding capacity is dependent on its carboxylic content.

The allergen-binding capacity of OC from different sources is superior to that of other biocompatible polysaccharides

The allergen-binding capacity of additional OC types from various sources was then evaluated, in which the homemade 16.3% carboxylated OC batch served as reference. As can be seen in Fig. 2*BII*, both a commercial cotton-derived OC (H-L 19.1%) and a regenerated OC (Reg 9.9%) that was prepared by us from regenerated cellulose demonstrated binding capacities similar to our 16.3% OC. In contrast, a commercial OC calcium salt (Ca-L 16.6%) with a similar carboxyl group content (Fig. 2*BII*) demonstrated 4- to 6-fold less allergen binding than the acidic noncharged OC types. In addition, unlike OC, chitosan and CMC, biocompatible polysaccharides that are commercially available, demonstrated negli-

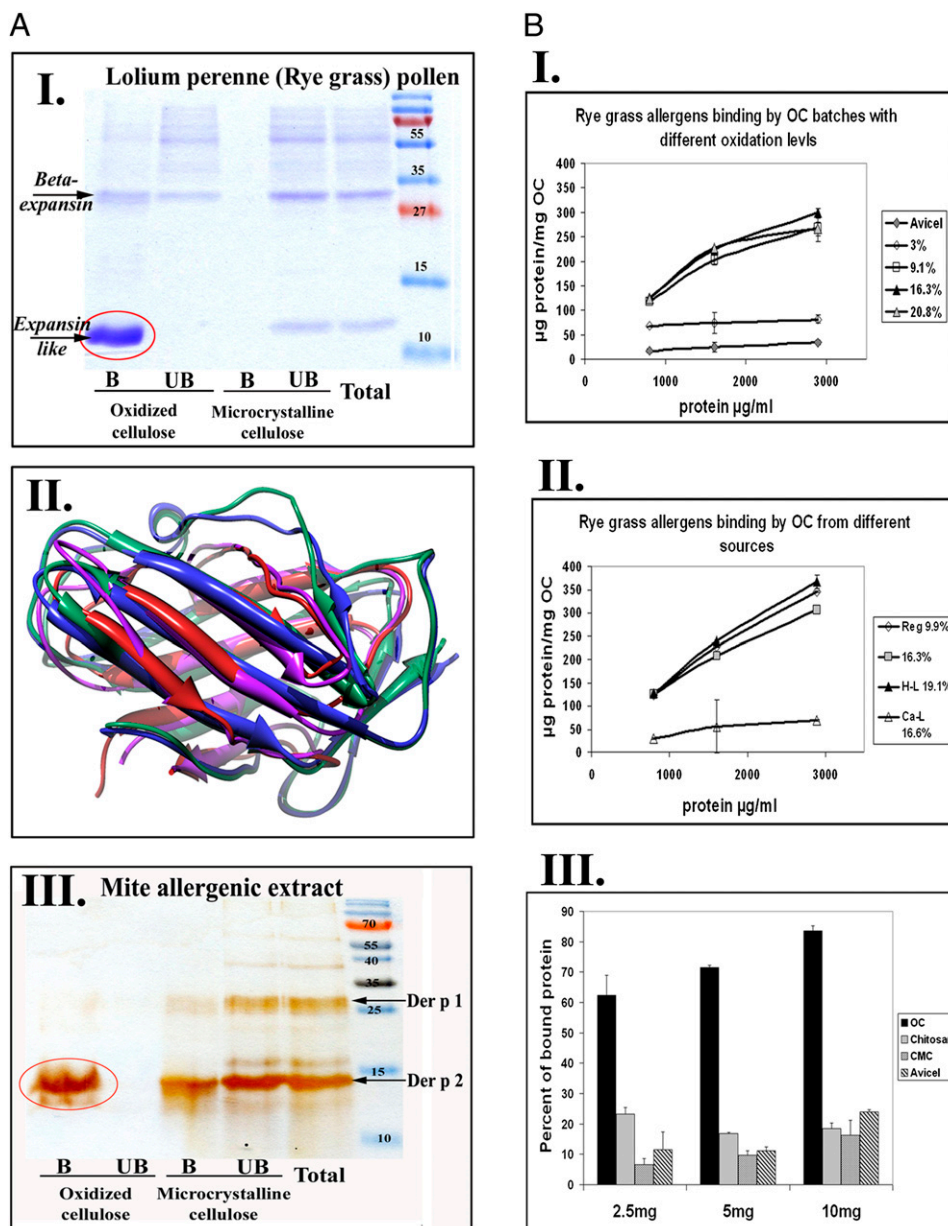
gible allergen-binding capacity (Fig. 2*BIII*). In conclusion, OC in its acidic uncharged form demonstrates potent allergen-binding capacities that are superior to any other polysaccharide that was tested, indicating its unique abilities as a proposed allergen blocker.

Pretreatment with OC inhalants inhibits the onset of allergic lung inflammation

As illustrated in Fig. 1*B*, pretreatment of OC powder inhalation prior to allergen exposure is predicted to prevent the initiation of allergy. To test this hypothesis, a rye grass-sensitized animal model was used. As anticipated, an airway challenge of *L. perenne* allergens in sensitized BN rats resulted in marked BAL eosinophilia (see Fig. 3*A*). However, allergic rats pretreated with an OC powder inhalant prior to the allergen challenge demonstrated reduced BAL eosinophilia with a marked reduction in the consequential allergic lung inflammation, as demonstrated by the decrease in their pathology scores (see Fig. 3*B*). Microscopic analysis of tissue sections demonstrated the decreased infiltration of inflammatory cells into the peribronchial tissues in allergen-challenged rat pretreated with OC (see Fig. 3*D*) as compared with allergen-challenged rats without OC pretreatment (see Fig. 3*C*).

To demonstrate that OC's ability to inhibit allergic reactions is dependent on its ability to specifically bind allergens, we used the OVA allergy animal model, which is induced by an isolated allergen and not a whole allergen extract. A pairwise structure analysis using secondary structure matching (SSM) (19) demonstrated similarity between the β -sandwich fold of the CBM2 family member Xylanase 10A (*Cellulomonas fimi*) and β sheet B of OVA (root mean square deviation [RMSD] = 3.13), suggesting an OVA polysaccharide-binding potential. As can be seen in Fig. 4*A*, OC indeed demonstrated profound affinity to OVA, in contrast to crystalline cellulose, which demonstrated only negligible al-

FIGURE 2. OC binding of CBM-containing allergens is more potent than any other polysaccharide examined, and is dependent on its level of oxidation and charge. Soluble *L. perenne*-derived pollen proteins (*AI*) or a mite allergen mix containing *D. pteronyssinus* and *D. farinae* (*AIII*) were incubated with oxidized or microcrystalline cellulose microparticles. Bound (*B*) and unbound (*UB*) fractions were separated by SDS-PAGE in parallel to total protein samples and detected with either Coomassie blue (*AI*) or silver (*AIII*) stains. Expansin and expansin-like (circled in red) proteins and mite Der p 1 and Der p 2 (circled in red) proteins were excised from the gel and identified by LC-MS/MS. A three-dimensional structure alignment of phl p I (β -expansin [purple]), phl p II (expansin-like [red]), Der f 2 (green), and Der p 2 (blue) was performed by the SSM service at the European Bioinformatics Institute (the RMSD of the structure analysis between Der f 2 and phl p I and II was 3.19 and 3.5, respectively) (19) (*AII*). Soluble *L. rigidum* pollen proteins were incubated with OC microparticles of varying oxidative degrees, or microcrystalline cellulose (Avicel), in which the amount of bound content was extrapolated from the unbound protein content (*BII*). Increasing concentrations of OC, microcrystalline cellulose (Avicel), CMC, or chitosan were used in a *L. perenne* allergen-binding assay (*BIII*).



lergen binding. The ability of OC to block OVA-induced immune responses was first examined in vitro by treating splenocytes from OVA-sensitized mice with either OVA only or an OC–OVA complex. As anticipated, treating the cells with OVA led to increased splenocyte proliferation, which was not seen when OC–OVA complexes were added to the cells (data not shown). Thus, OC binding to OVA was able to block its immunogenic potential. As in the grass allergen animal model, pretreatment of OVA-sensitized BALB/c mice with an OC powder inhalant prior to OVA allergen challenge led to 10-fold reduction in measured BAL eosinophilia (see Fig. 4B). Additionally, a marked decrease in expression of the Th2-related cytokines IL-4 and IL-5 was observed in OC-treated mice (see Fig. 4C, 4D, respectively), linked with more favorable pathology scores of the lung (see Fig. 4E). OVA-sensitized animal groups demonstrated high levels of both anti-OVA IgG and IgE as compared with untreated naive mice (Fig. 4F, 4G, respectively). However, the additional increase in anti-OVA Ab levels that was induced by OVA airway challenges was blocked by the pretreatment with OC (Fig. 4F, 4G). Microscopic analysis of lung sections demonstrated decreased

infiltration of inflammatory cells into the peribronchial tissues in allergen-challenged mice pretreated with OC (see Fig. 4I) as compared with mice treated with an allergen challenge only (see Fig. 4H). In conclusion, pretreatment with OC microparticle inhalant was demonstrated to inhibit allergic lung inflammation in two different animal models that are induced by different allergens, suggesting a potential use of OC in the treatment of allergy and asthma.

OC is efficiently cleared from the airways and metabolized in the body

Although it is approved for use in humans as a surgical hemostat, OC safety and clearance from the airways have never been tested. To this end, OC microparticles ($\sim 10 \mu\text{m}$) were fluorescently labeled with Cy5.5 and intranasally administered to mouse, and their clearance was followed via in vivo imaging. OC was detectable in the nose, tracheal area, and the left lung at 40 min postadministration (Fig. 5AII). However, OC was almost completely cleared from the upper airways and was found in large quantities in the intestines within 4 h of administration (Fig.

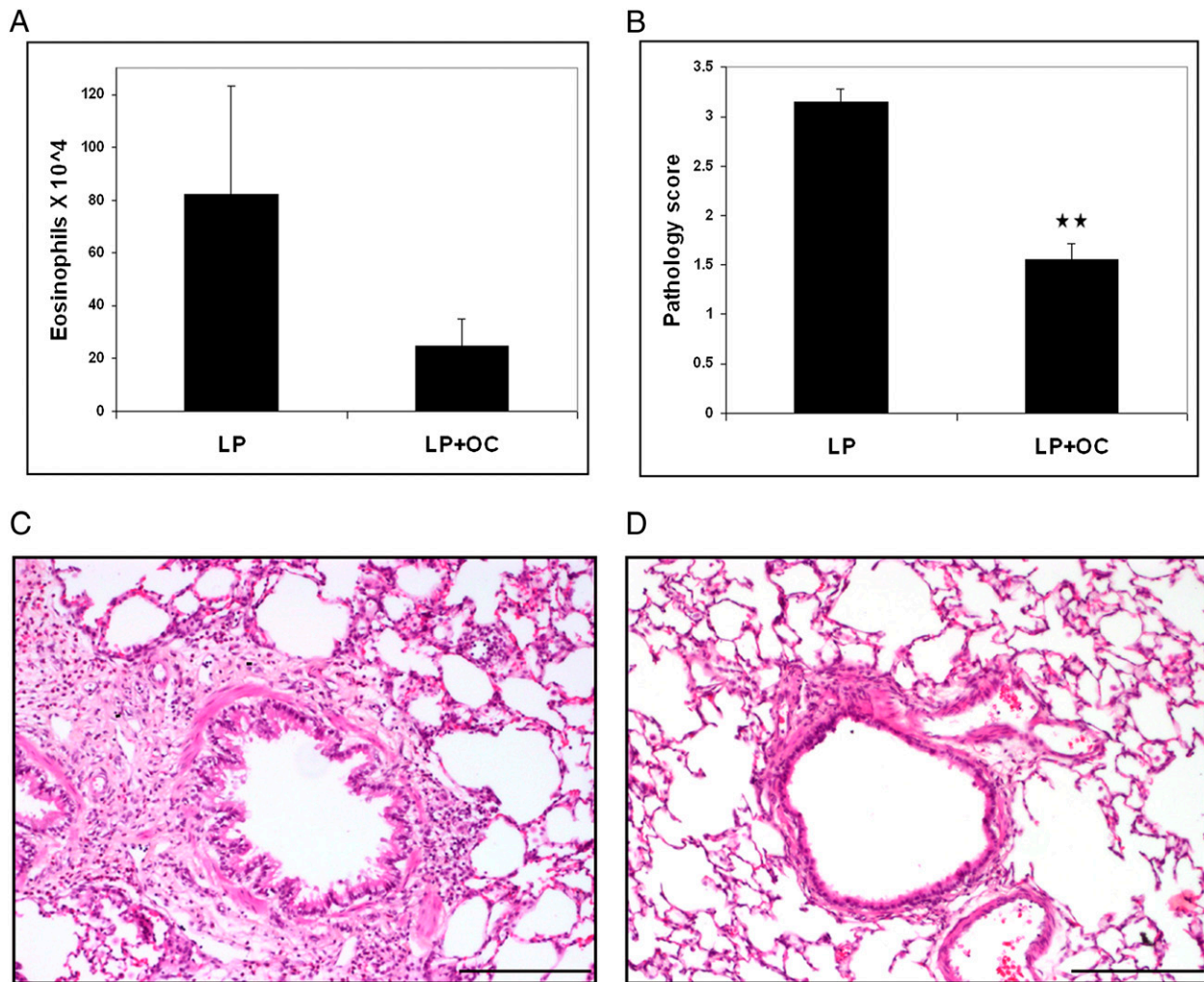


FIGURE 3. Inhibition of allergic lung inflammation by pretreatment with an OC microparticle inhalant in a rat model of grass pollen-induced asthma. Sensitized BN rats were challenged with an inhalation of *L. perenne* pollen proteins suspended in PBS (LP). In parallel, a group of BN rats was pretreated with an OC powder inhalant prior to *L. perenne* allergen challenge (LP + OC). Rats were sacrificed 48 h postchallenge, and BAL fluid eosinophil counts were recorded (A). Lungs were then fixed, followed by H&E staining. Lung section pathology scores were evaluated (B), and representative sections were photographed: *L. perenne* allergen challenge only (C) or pretreatment with OC powder inhalation, followed by *L. perenne* allergen challenge (D). Original magnification $\times 200$; scale bar, 200 μm . $n = 5$ rats/group. ** $p < 0.01$.

5AIII). As intranasal administration of OC suspensions resulted in OC overdose in the lungs, it allowed us to follow OC clearance from the lungs over time. As can be seen in Fig. 5BII and 5BIII, OC was almost completely cleared from the lungs within 13 d and was completely cleared within 1 mo, demonstrating effective clearance even at overdose OC quantities. To assess OC metabolism after its clearance from the respiratory system, the liver, kidney, and spleen were removed from mice at different time points following intranasal OC administration for fluorescence evaluation. OC was already detected in high quantities in the liver and kidney, but not in the spleen, 7 h after its administration (Fig. 5CII). OC was consistently found in the liver and kidney over the 13 d following administration (Fig. 5CIII [72 h], 5CIV [13 d]) and mirrored the gradual decrease in pulmonic OC levels, indicating continuous OC clearance from the lungs, but no accumulation in these organs. Fluorescence levels in the liver and kidneys returned to close to basal levels by day 13 (compare Fig. 5CI with 5CIV).

Long-term administration of OC to Wistar rats does not cause pulmonary toxicity

Because OC inhalations are being suggested for application as a daily prophylactic treatment for asthma, we next assessed its

safety as an extended-use inhalant. Wistar rats were subjected to a high dose of OC particle powder inhalation for a period of 21 d and were compared with untreated age-matched rats. OC-treated rats gained weight throughout the experiment at the same rate as control rats (Fig. 5DI, 5DII) and did not develop any pathological defects in the lungs (Fig. 5DIII, 5DIV, 5DV, and 5DVI). Thus, it can be concluded that OC is effectively cleared from the lungs and other organs and does not induce toxicity upon its long-term administration by inhalation.

Discussion

In our study we show that OC, a compound commonly used as an absorbable surgical hemostat, bears substantial binding capacities toward grass pollen allergens from groups I (β -expansin), II, and III (expansin-like proteins), and to mite Der f 2 and Der p 2 allergens that surpassed the ability of all the other polysaccharides examined. Because we were able to show the CBM portion of expansins share structural homology with Der f 2 and Der p 2, we speculate this potent binding is initiated by these CBM-like structures. In addition, sharp reductions in eosinophilia and lung pathology scores were recorded upon pretreatment of sensitized animals with an OC inhalant prior to allergen challenge. In vivo imaging of OC ad-

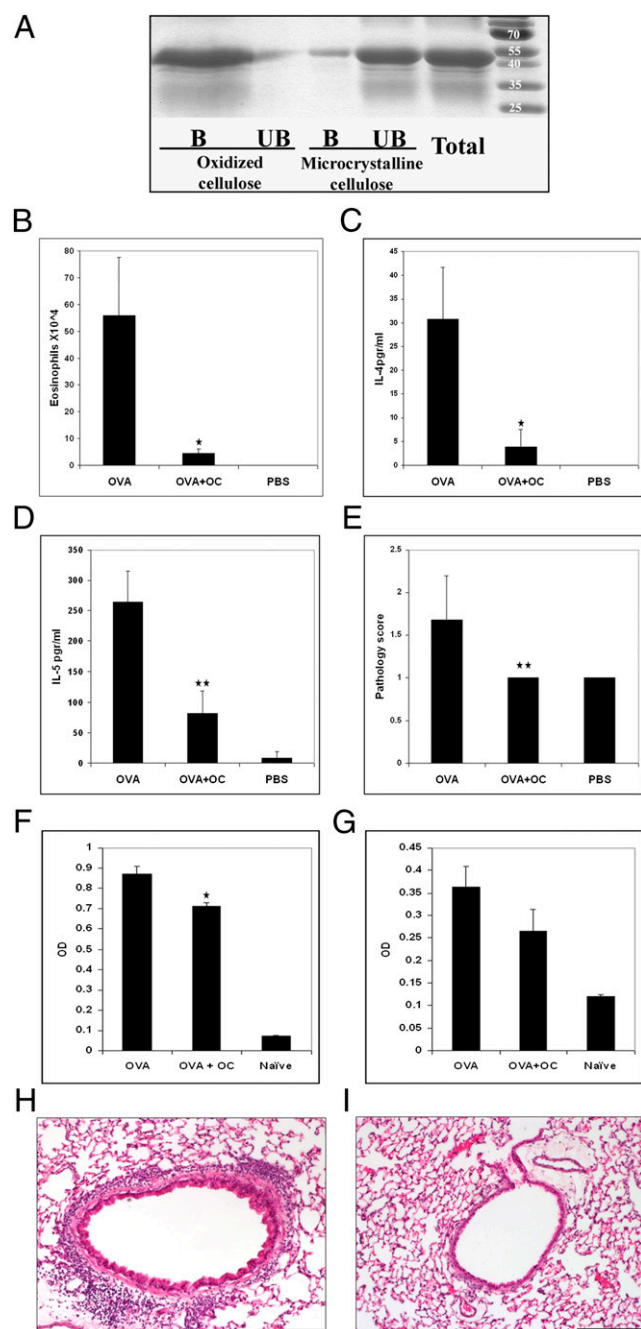


FIGURE 4. Inhibition of allergic lung inflammation by pretreatment with an OC microparticle inhalant in a mouse model of OVA-induced asthma. OVA was incubated with microparticles of either oxidized or microcrystalline cellulose. Bound (B) and unbound (UB) fractions and total protein samples were separated by SDS-PAGE and detected using Coomassie dye (A). Sensitized BALB/c mice were challenged by inhalation of either OVA or PBS, or pretreated with an OC powder inhalant prior to OVA allergen challenge (OVA+OC). Mice were sacrificed 48 h postchallenge, and BAL fluid eosinophil counts (B) and IL-4 (C) and IL-5 (D) levels were assessed. Lungs were then fixed and stained with H&E. Lung section pathology scores were evaluated (E), and representative lung sections were photographed: OVA challenge only (H) and OVA + OC (I). Levels of soral OVA-specific IgG and IgE Abs (F and G, respectively) were determined by ELISA. Original magnifications $\times 200$; scale bar, 200 μm . * $p < 0.05$; ** $p < 0.01$, between the OVA group and the OVA + OC group. $n = 4-8$ mice/group.

ministrated to the airways demonstrated its effective clearance from the airways and lungs. Moreover, 21 d of OC powder inhalations did not cause toxicity to the lungs, suggesting that the use of an OC

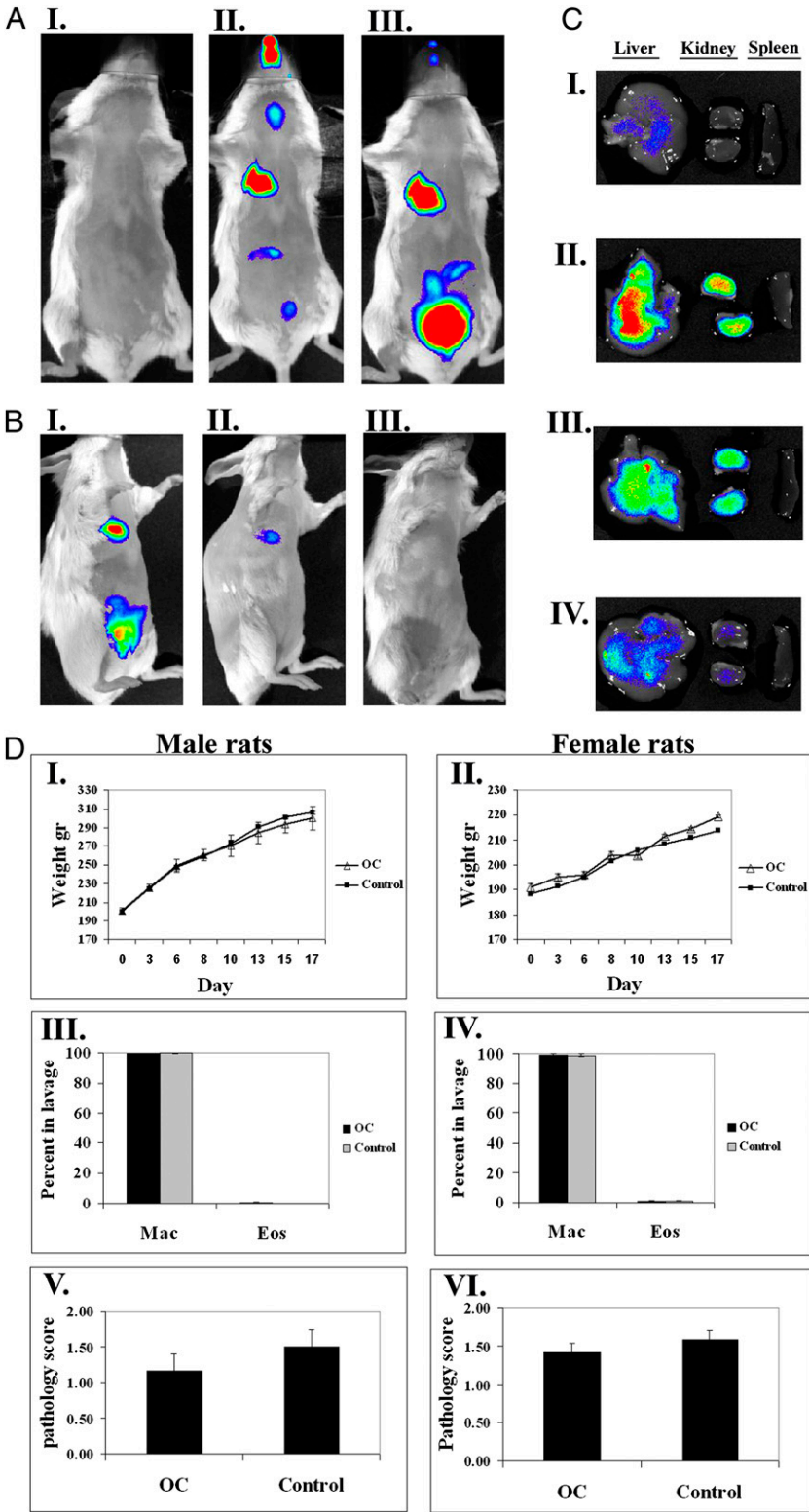
inhalant is safe. We propose that OC allergen binding promotes the clearance of allergens from the airways by the mucociliary system prior to the induction of an allergic reaction.

Pathogens frequently present surface carbohydrate-binding proteins that mediate recognition of and attachment to host cells (20). Thus, we hypothesize that the aggressive host immunogenic responses to CBM-containing allergens may arise from defense mechanisms originally directed against similar structures of pathogenic Ags. We have demonstrated that OC preferably binds the Der f 2 and Der p 2 mite allergens (Fig. 2AIII), both of which contain a CBM closely resembling that of expansins (Fig. 2AII). In support of these findings and hypothesis, the Der f 15 and Der f 18 central dog mite allergens that have been recently proven to also be potent human allergens (21) contain a chitin binding domain (CBM14) (12, 13). In addition, Ole e 10, a key olive allergen, was shown to preferentially bind 1,3- β -glucans and is a member of the new CBM family 43 (14). Furthermore, the hev b 6.01 latex allergen (also termed prohevein) contains a CBM family 18 chitin binding domain known as the hevein domain, which serves as the protein's main IgE-binding epitope. Moreover, a group of class I chitinase fruit allergens (kiwi, banana, avocado, chestnut, and others) contains hevein-like chitin binding domains, leading to cross-reactivity between latex and food allergens responsible for the latex-fruit syndrome (11). Thus, the CBM is proposed to play a central role in the induction of various allergic conditions.

The immunogenic potential of an inhaled allergen is highly dependent on its ability to breach the epithelial barrier of the airway and to reach intraepithelial mast cells and dendritic cells (Fig. 1A). Many potent allergens contain intrinsic protease activity that facilitates their penetration through the intraepithelial layer (22, 23). The CBM of CBM-containing proteins have been shown to disrupt hydrogen bonding between polysaccharides in a non-hydrolytic manner (9). β -expansin has been proposed to be involved in pollen penetration and germination through the pistil (9). Swollenin, a *Trichoderma reesei*-derived fungal expansin-like protein, was found to increase fungus plant root invasion and colonization competence in a CBM-dependent manner (24). Moreover, enhanced surface expression of galectin-3, a β -galactoside-binding animal lectin, has been correlated with increased tumor invasiveness and metastatic indices (25). Thus, ability of carbohydrate-binding proteins to induce allergic reactions may be due to promoted proficiency in breaching the epithelial barrier. OC-allergen CBM binding may inhibit allergen penetration across the epithelial barrier and the induction of allergic reactions.

It has been shown previously that expansin binding in plants is mediated via the less crystalline and more amorphous cell wall components (26), as demonstrated in this study through its preferential binding to OC, which is far more amorphous than cellulose (see Fig. 2AI). The amorphous and structurally plastic OC polysaccharide contains a large surface area supportive of high quantities of both specific and nonspecific interactions. Thus, it is suggested that the high allergen-binding capacity of OC is mediated both by its large surface area that allows the initiation of nonspecific interactions with different allergens and via its specific interactions with CBMs. The interactions between pollen proteins and OC reported in this study were not mediated by the negative charge of OC carboxylic groups; OC calcium salt in which the carboxylic groups are negatively charged demonstrated a reduced allergen-binding capacity compared with the potent binding capacity of OC in its acidic form in which the carboxylic groups are generally uncharged. The unique ability of acidic OC to efficiently bind allergens was further demonstrated in comparison with a wide variety of polysaccharides, including cellulose, CMC, and chitosan, which demonstrated poor allergen binding.

FIGURE 5. OC is efficiently cleared from the lungs and airways, and does not induce pulmonic toxicity. A suspension of OC-Cy5.5 was intranasally administered to mice, and its distribution was monitored over time (A–C). Near-IR in vivo images of a mouse before OC-Cy5.5 administration (AI [background image]), 40 min postadministration (AII), and 4 h postadministration (AIII) are presented. The clearance of OC-Cy5.5 from the lung was monitored by near-IR in vivo imaging at 40 min postadministration (BI), 13 d postadministration (BII), and 28 d postadministration (BIII). Livers, kidneys, and spleens were removed from untreated mice (CI [background fluorescence]), 7 h after OC-Cy5.5 administration (CII), 72 h postadministration (CIII), and 13 d postadministration (CIV). Male (DI, DIII, DV) or female (DII, DIV, DVI) Wistar rats were either treated with OC powder inhalation for a period of 21 d or left untreated. Rat weights were recorded periodically throughout the 21-d period (DI and DII). At day 21, rats were sacrificed and BAL fluid differential cell counts were performed after May-Grunwald Giemsa staining (DIII, DIV). Lungs were then fixed, followed by H&E staining. Lung section pathology scores were evaluated (DV, DVI).



To date, asthma treatment is based on the combination of anti-inflammatory drugs, mainly corticosteroids (27). Whereas inhalant corticosteroid use is relatively safe, long-term exposure can lead to adverse side effects. Application of the OC microparticle-based allergen-quenching treatment may reduce or eliminate the need for corticosteroid treatment. OC bears an impressive safety record as a surgical hemostat (28–30) and lacks active substances, suggesting that it will not cause any adverse effects.

Although OC features an impeccable safety record for in vivo application as a hemostatic agent (30), OC airway safety has never been demonstrated. Particle toxicity to the lungs and airways is highly influenced by its cytotoxicity, immunogenic potential, and biopersistence, or resistance to degradation in the lungs (31). Despite its extensive biopersistence, little evidence exists of cellulose dust-related toxicity in the paper and wood industries. Thus, OC, which has been proven to be both biocompatible and bio-

adsorbable (28, 29, 32), will presumably be well tolerated in the airways and lungs. Although it is assumed that OC particles that reach the deeper regions of the lungs will dissolve, degrade, and undergo macrophagial clearance (33), we propose use of 5–15 μM OC particles to allow for their deposition in the extrathoracic and upper bronchial regions, to further decrease risk of toxicity and enhance airway clearance (34). Our data derived from in vivo imaging of fluorescently labeled OC deposited in the lungs of mice indicate that OC is effectively cleared from the lungs and airways (Fig. 5A, 5B), later to be metabolized and fully cleared from the body within 13 d of administration (Fig. 5C). The efficient clearance of OC from the airways (Fig. 5A), together with the ability of the OC inhalant to inhibit allergic reactions in vivo (Figs. 3, 4), supports our proposal of mucociliary-based clearance of OC–allergen complex from the airways (Fig. 1). In addition, high doses of OC inhalation administered over a period of 21 d were not toxic to the lungs and airways (Fig. 5D).

The efficacy of allergen-induced bronchitis prevention by OC was demonstrated in two allergen-sensitized animal models, known to be less sensitive than humans. Due to the reduced allergen sensitivity in animal models compared with atopic individuals, high quantities of allergens were required to induce allergic lung inflammation in animal models. In fact, the allergen concentrations present in the inhalation chamber used in this study were at least 500-fold higher in the BN model and 25,000-fold higher in the BALB/c model than the reported environmental 2,000 grain/ m^3 grass pollen concentrations at the peak of allergen season (35). Thus, we predict clinically relevant OC–allergen capturing efficacy in allergic human individuals who are typically exposed to dramatically lower allergen concentrations than those applied in this study. In conclusion, the significant binding capacity of OC to various allergens holds promise for an alternative, safe, and effective treatment for allergic individuals.

Acknowledgments

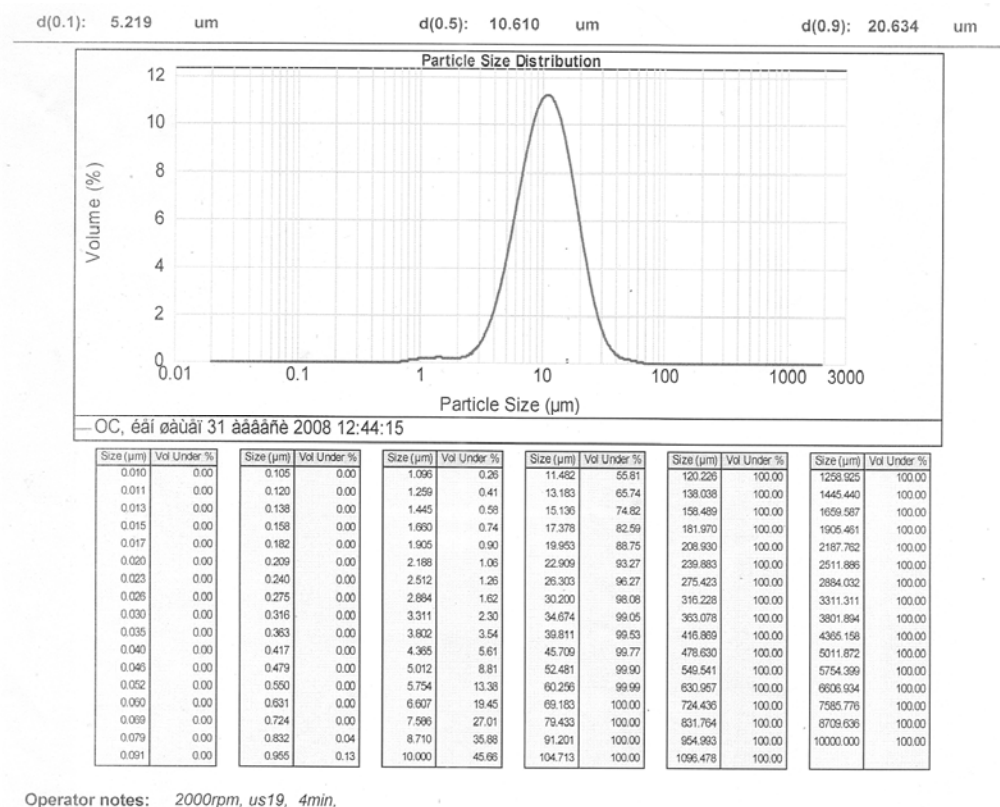
We thank Prof. Dov Zipori from the Department of Molecular Cell Biology (Weizmann Institute of Science, Rehovot, Israel) for generous help during this study.

Disclosures

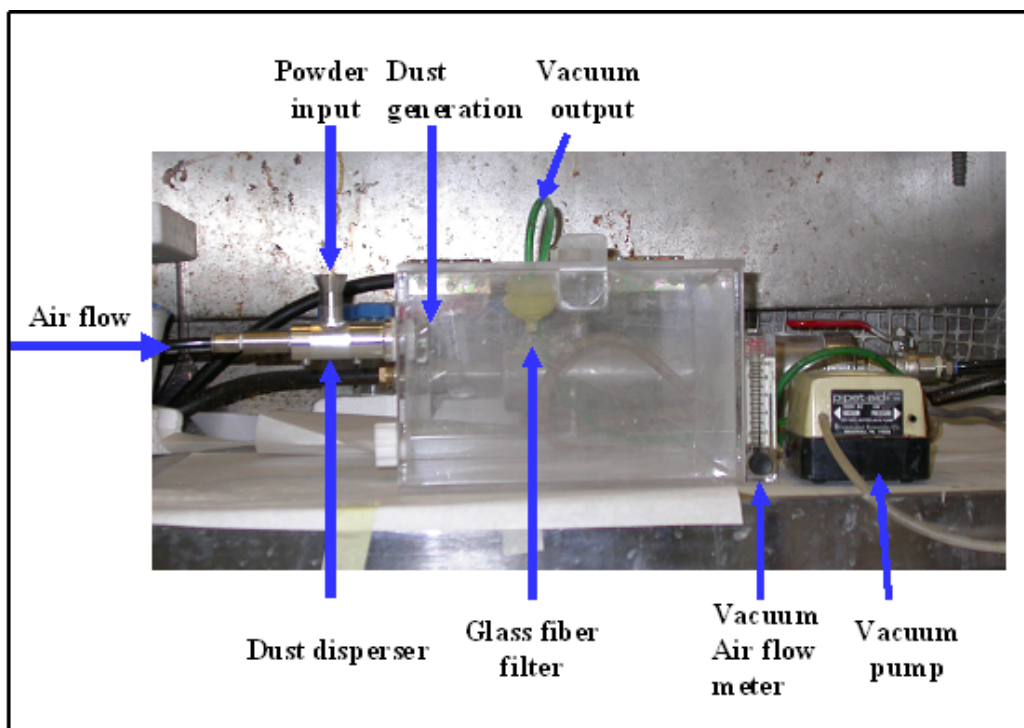
The authors have no financial conflicts of interest.

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Supplementary FIGURE 1. Particle size distribution of milled OC particles as determined by MASTERSIZER 2000.



Supplementary FIGURE 1

Supplementary FIGURE 2. Dust disperser

The dust disperser that can be seen at supplementary Fig. 1, connected to the container on the right, was developed by Super Fine LTD, Yokneam, Israel. The dust disperser is connected to an air flow of constant pressure. Micronized powder of oxidized cellulose (OC (200mg) is poured into the funnel and the powder is immediately dispersed in the container (the air flow is turned off as soon as the powder is dispersed). Oxidized powder (200mg) is repetitively dispersed every 0.5 minute for continuous time periods.