

Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation

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Background: Sublingual immunotherapy is a noninvasive and efficacious treatment of type I respiratory allergies. A murine model of sublingual immunotherapy is needed to understand better the immune mechanisms involved in successful immunotherapy and to assess second-generation candidate vaccines.

Objective: Herein, we developed a therapeutic murine model of sublingual immunotherapy in which we document the value of mucoadhesive formulations to enhance treatment efficacy.

Methods: BALB/c mice were sublingually treated with soluble or formulated ovalbumin before or after sensitization with ovalbumin. Airways hyperresponsiveness and lung inflammation were assessed by whole-body plethysmography and lung histology, respectively. Humoral and cellular immune responses were monitored by ELISA and ELISPOT techniques. **Results:** Prophylactic sublingual administration of ovalbumin completely prevents airways hyperresponsiveness as well as IL-5 secretion and IgE induction. Therapeutic administration of ovalbumin as a solution via either the sublingual or oral route has a limited efficacy. In contrast, sublingual application of ovalbumin formulated with maltodextrin to enhance mucosal adhesion results in a major reduction of established airways hyperresponsiveness, lung inflammation, and IL-5 production in splenocytes. This mucoadhesive formulation significantly enhances ovalbumin-specific T-cell proliferation in cervical but not mesenteric lymph nodes, and IgA production in the lungs. **Conclusion:** A mucoadhesive maltodextrin formulation of ovalbumin enhances priming of the local mucosal immune system and tolerance induction via the sublingual route. **Clinical implications:** Mucoadhesive formulations offer the opportunity to improve dramatically sublingual immunotherapy in human beings, most particularly by

simplifying immunization schemes. (*J Allergy Clin Immunol* ■■■■;■■■:■■■-■■■.)

Key words: Allergy vaccines, mucosal formulation, sublingual immunotherapy

Allergen-specific sublingual immunotherapy (SLIT) is an efficient treatment of type I respiratory allergies to a variety of allergens.¹⁻³ However, many areas of investigation remain to improve SLIT further, relating for example to immune effector mechanisms involved,^{4,5} or to the determination of optimal dosing regimens and treatment schedules.⁶ Also, the use of adjuvants as well as mucosal formulations to improve current sublingual vaccines has been totally unexplored. Whereas various murine models are available to test new strategies of allergen-specific immunotherapy conducted via the parenteral, nasal, or oral routes,⁷⁻¹¹ it has been a challenge to develop such an animal model for sublingual immunization, largely because of difficulty controlling and documenting the level of antigen exposure to the sublingual immune system.

In the current study, we tested various prophylactic or therapeutic sublingual immunization approaches in BALB/c mice with ovalbumin (OVA)-induced asthma. Specifically, we explored means to enhance adhesion of the antigen/allergen to the sublingual mucosa and to facilitate capture by the local immune system. We report herein on the capacity of such a mucoadhesive antigen formulation based on polymerized maltodextrins (1) to prime the local immune system better and (2) to enhance dramatically SLIT efficacy.

METHODS

Animals, culture medium, reagents, and formulations

Six-week-old female BALB/c mice were purchased from Charles River (L'Arbresle, France) and maintained on an OVA-free diet. Each treatment group was composed of 8 mice, with a group of 4 healthy untreated mice as a control. DO11.10 female mice transgenic for OVA-specific T-cell receptor have approximately 50% of their CD4⁺ T cells expressing a T-cell receptor specific for the peptide 323-339 fragment of OVA.¹² International levels of ethical standards for the manipulation of animals were applied.

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Abbreviations used

AHR: Airway hyperresponsiveness
 CFSE: Carboxy-fluorescein diacetate succinimidyl ester
 FACS: Fluorescence-activated cell sorter
 HES: Hematoxylin, eosin, and safran
 LN: Lymph node
 OVA: Ovalbumin
 Penh: Enhanced pause
 PSC: Polysaccharidic formulated core
 SFC: Spot-forming cell
 SLIT: Sublingual immunotherapy
 UK: United Kingdom

Complete medium for spleen cells culture consisted of Ultraculture medium (Cambrex, Verviers, Belgium) and 1% L-glutamine (Invitrogen, Carlsbad, Calif). OVA grade V was purchased from Sigma Chemicals Aldrich (Saint-Quentin en Fallavier, France). All OVA batches used in the current study were confirmed to contain less than 100 UI/mL endotoxin by the Limulus amoebocyte lysate assay.

In selected experiments, OVA was adsorbed on a Delstrip 3-mm thin cellulose film (a gift from Cardinal Health, Wiltshire, United Kingdom [UK]) by spotting 500 μ g (ie, 5 μ L of a 100 mg/mL OVA solution). Polysaccharidic formulated core (PSC) OVA was prepared as described elsewhere.¹³ Briefly, 100 g maltodextrins (GLUCIDEX, Roquettes Frères, Lestrem, France) were suspended in 200 mL 2 mol/L NaOH at room temperature. Maltodextrins were then cross-linked by the addition of 4.7 mL 2,3-epoxychloropropane and branched with 31.2 g glycidyl trimethyl ammonium. The resulting gel was homogenized at high pressure to give a suspension of 60 nm cationic nanoparticles, further purified by ultrafiltration (Minisette system, 30 kD membrane; Pall, Saint-Germain-en-Laye, France) and microfiltration (spiral cap, 0.2 μ m; Pall). OVA was mixed with such premade PSC nanoparticles, and antigen binding was estimated to be almost complete by surface plasmon resonance analysis.¹⁴

Animal sensitization and desensitization

Sensitization was performed by 2 intraperitoneal injections at 14-day intervals with 10 μ g OVA adsorbed on 2 mg Al(OH)₃ administered in a volume of 100 μ L. This was followed by a 20-minute aerosol challenge with 1% wt/vol OVA on 4 consecutive days using an aerosol delivery system (Buxco Europe Ltd, Winchester, UK), as shown in the experimental design in Fig 1, A. Control animals were treated with sterile PBS by using the same protocol. For sublingual treatment before sensitization (prophylaxis; Fig 1, B), OVA was applied as a solution (500 μ g in 20 μ L PBS) twice a week for 3 weeks under the tongue while holding animals on their back (for 1 minute) to prevent deglutition. Control animals were sham-treated with PBS before sensitization. For sublingual treatment after sensitization (therapy; Fig 1, C), OVA was applied as a solution (500 μ g in 20 μ L PBS) as described, or in a mucoadhesive form (Delstrip-OVA or PSC-OVA, using 500 μ g or 50 μ g OVA per dose) twice a week for 2 months. Control animals were sham-desensitized with sterile PBS, Delstrip, or PSC formulations alone. For oral immunotherapy, OVA (500 μ g in 100 μ L PBS) was administered by intragastric gavage. Two days after treatment, mice were challenged with OVA aerosols (1% wt/vol) on 2 consecutive days. An overview of group characteristics for therapeutic SLIT is presented in Table E1 (see this article's Online Repository at www.jacionline.org).

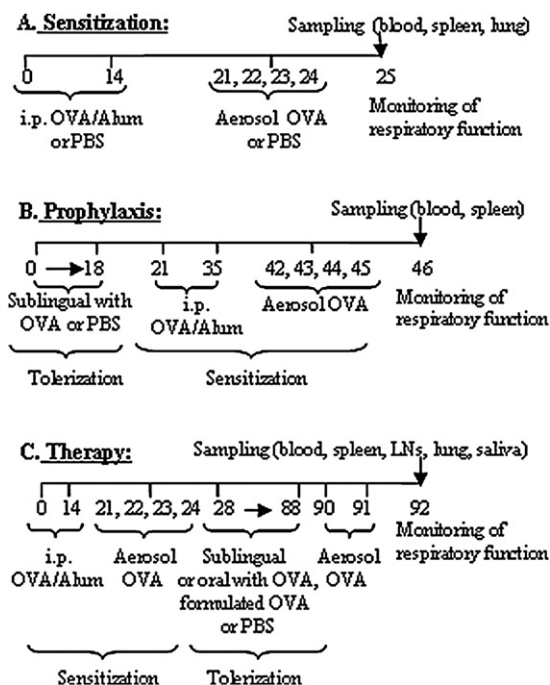


FIG 1. Experimental design and sampling. All mice were sensitized by intraperitoneal injections with OVA/alum followed by an aerosol challenge (sensitization, **A**). Before (prophylaxis, **B**) or after (therapy, **C**) sensitization, BALB/c mice were sublingually treated with soluble or formulated OVA. Measurements of AHR were performed by whole-body plethysmography.

Determination of airway hyperresponsiveness and lung histology

Measurements of airway hyperresponsiveness (AHR) were performed 24 hours after the last challenge by whole-body plethysmography (Buxco Europe Ltd, Winchester, UK).¹⁵ Airway resistance was expressed as enhanced pause (Penh). A Penh index, expressed as an increase relative to the baseline airway resistance, was obtained by dividing the Penh measured after exposure to a given concentration of inhaled methacholine with the Penh measured after inhalation of nebulized PBS. The Penh index at 50 mg/mL metacholine was recorded and plotted as reported elsewhere.¹⁶

For tissue histology, mice were killed by cervical dislocation. Lungs were recovered and fixed in phosphate buffered formalin-zinc and embedded in paraffin wax. Sections were stained with hematoxylin, eosin, and safran (HES) for the determination of cellular infiltrates and Alcian blue to identify mucus-producing goblet cells in the epithelial border. A semiquantitative assessment of both perivascular inflammation and eosinophil infiltration was performed on coded samples. Normal lung (no inflammation) was given a (–) score. A mild (+) score was assigned to multifocal perivascular infiltrates. A moderate score (++) was given to sections with more disseminated infiltrates. A marked (+++) score was given to sections with marked and diffuse perivascular inflammation. For the PSC and PSC-OVA groups, eosinophil counts were estimated for each mouse at a 40-fold magnification in 10 fields randomly selected across the lung. Results were expressed as the mean counts $\pm$ SEMs from 8 mice per group.

Determination of allergen-specific antibody levels

Blood samples were collected at the retroorbital sinus to assess OVA-specific antibody levels by ELISA (compare sampling, Fig 1).

For detection of IgG₁ and IgG_{2a} antibodies, purified OVA (0.2 µg) was coated overnight at 4°C onto ELISA plates (Nunc, Roskilde, Denmark). After washing and blocking steps, mouse sera dilutions (1/100 to 1/12800 for IgG₁; 1/20 to 1/2560 for IgG_{2a}) were incubated for 1 hour at 37°C. Plates were washed and biotinylated-rat antimouse IgG₁ or IgG_{2a} antibodies (dilution 1/100; BD Pharmingen, San Jose, Calif) were added for 1 hour at 37°C. Streptavidin peroxidase-conjugated rat antimouse IgG antibodies (dilution 1/400; BD Pharmingen) were used for detection with orthophenylenediamine as a substrate (Sigma Chemicals Aldrich). The reaction was stopped with 2 mol/L sulfuric acid, and optical densities were determined by using an ELISA plate reader at 492 nm (Labsystems, Helsinki, Finland).

For detection of IgE antibody titers, antimouse IgE antibodies (1 µg/well; Bethyl Laboratories, Montgomery, Tex) were coated onto ELISA plates. After washing and blocking steps, mouse sera dilutions (1/20 to 1/2560) were incubated for 1 hour at 37°C. OVA-digoxigenin was incubated (at a 1/80 dilution) for 1 hour at 37°C, and horseradish peroxidase-conjugated rabbit antidigoxigenin serum (Roche) was used for detection at a 1/1000 dilution. A 2,2'-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) substrate was added (Roche). Optical densities were measured by using an ELISA plate reader at 405 nm.

Antibody titers were defined as the reverse of the last dilution for which the optical density value was 2-fold over background.

To determine allergen-specific antibody levels at mucosal surfaces, 1 lobe was recovered from the lung of each mouse (compare sampling, Fig 1, C). Tissues from 8 mice were pooled and teased in a complete medium, and cell suspensions were filtered through a 297-µm cell dissociation sieve (Sigma Chemicals Aldrich). The recovered cell suspensions were centrifuged at 200g for 5 minutes at room temperature. Supernatants were collected to perform IgA level determination by ELISA. Briefly, microplates were coated with a goat antimouse IgA (0.1 µg/well, Bethyl Laboratories) and washed, and supernatant dilutions (1/10 to 1/1280) were incubated for 1 hour at 37°C, followed by OVA-digoxigenin (dilution 1/80). Plates were washed, and horseradish peroxidase-conjugated rabbit antidigoxigenin (Roche) was used for detection, as described.

Assessment of cellular immune responses by ELISPOT and ELISA

Spleens were removed (compare sampling, Fig 1), and splenocytes were isolated and plated in triplicate at 1×10^6 cells per well in 96-well flat-bottom ELISPOT plates (Millipore Corp, Bedford, Mass). Stimulation conditions used were medium alone, OVA (100 µg/mL), or phorbol 12-myristate 13-acetate (50 ng/mL; Sigma Chemicals Aldrich) plus ionomycin (500 ng/mL, Sigma) as a positive control. Plates were incubated for 72 hours at 37°C in 5% CO₂/95% air. Spot-forming cells (SFCs) were monitored for both IFN-γ and IL-5 production by using ELISPOT kits (R&D Systems, Minneapolis, Minn) as per the manufacturer's instructions and assessed with an ELISPOT reader (Carl Zeiss Microimaging, GmbH, Göttingen, Germany). IL-10 was measured in supernatants of spleen cell cultures (1×10^6 cells per well) by using an ELISA kit (R&D Systems) as per the manufacturer's recommendations.

Adoptive T-cell transfer and proliferation assay

CD4⁺ T cells were purified out of spleens from DO11.10 mice by magnetic bead separation using the mouse CD4 negative isolation kit according to the manufacturer's instructions (Invitrogen). The resulting T-cell preparations contained 95% to 99% CD4⁺ T cells, as confirmed by fluorescence-activated cell sorter (FACS) analysis. DO11.10 CD4⁺ T cells were labeled with 1 µmol/L carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Ore) for 5 minutes at 37°C in PBS. After 2 washes, 5×10^6 cells were

adoptively transferred by retro-orbital intravenous injection into BALB/c mice at day 0. Twenty-four hours later, mice (4 mice per group) were treated by the sublingual route with either soluble OVA (500 µg in 20 µL PBS) or PSC-OVA (500 µg OVA per dose). Control animals were treated with either sterile PBS or the PSC formulation alone. To measure T-cell proliferative responses *in vivo*, cervical/maxillary and mesenteric lymph nodes (LNs) were recovered at day 10 and pooled within each group of 4 mice. OVA-specific T cells were stained with the anticonotypic phycoerythrin-KJ1.26 mAb (BD Biosciences, San Jose, Calif), and proliferating cells were evaluated by FACS analysis (FC500 Flow Cytometer; Beckman Coulter, Villepinte, France) as cells with decreased fluorescence.

Statistical analysis

Mean comparisons were performed by using the Student *t* test. A *P* value < .05 was considered the limit of statistical significance.

RESULTS

Clinical and immunologic assessment of OVA-sensitized mice

Mice sensitized to OVA (Fig 1, A) were analyzed for signs of airways inflammation as well as humoral and cellular immune responses. OVA-sensitized mice exhibit a readily detectable AHR in response to methacholine (50 mg/mL; Fig 2, A) as well as a major bronchial inflammation associated with both a massive cellular infiltration and mucus hypersecretion (Fig 2, B) compared with PBS-treated control mice. The sensitization protocol induces high OVA-specific IgG₁ and IgE but low IgG_{2a} serum antibody levels (Fig 2, C). In accordance with this pattern of antibody responses, OVA-specific splenocytes from sensitized animals produce high levels of IL-5 and IL-10 (Fig 2, D). No IFN-γ or TGF-β was detected after stimulation of splenocytes with OVA (data not shown). Thus, OVA-sensitized mice exhibit a bona fide T_H2 immune response associated with airways inflammation.

Prophylactic sublingual administration of OVA prevents AHR, IL-5 secretion, and IgE induction

We first assessed in this model the capacity of sublingual immunization to prevent the development of allergic immune responses. To this aim, OVA was administered sublingually as a solution (500 µg/dose) twice a week for 3 weeks before sensitization (prophylaxis, Fig 1, B). Results shown in Fig 3 are representative of 3 independent experiments. As indicated, prophylactic sublingual administration of OVA almost totally prevented AHR as shown by Penh measurement, yielding values comparable to healthy (ie, nonsensitized) animals (Fig 3, A). In addition, SLIT prophylaxis very efficiently prevented both IL-5 production by OVA-specific spleen cells (Fig 3, B) and OVA-specific IgE secretion (Fig 3, C).

Therapeutic sublingual administration of formulated OVA reduces established AHR and bronchial inflammation

Because allergy vaccines are administered therapeutically, we assessed SLIT efficacy in animals with an

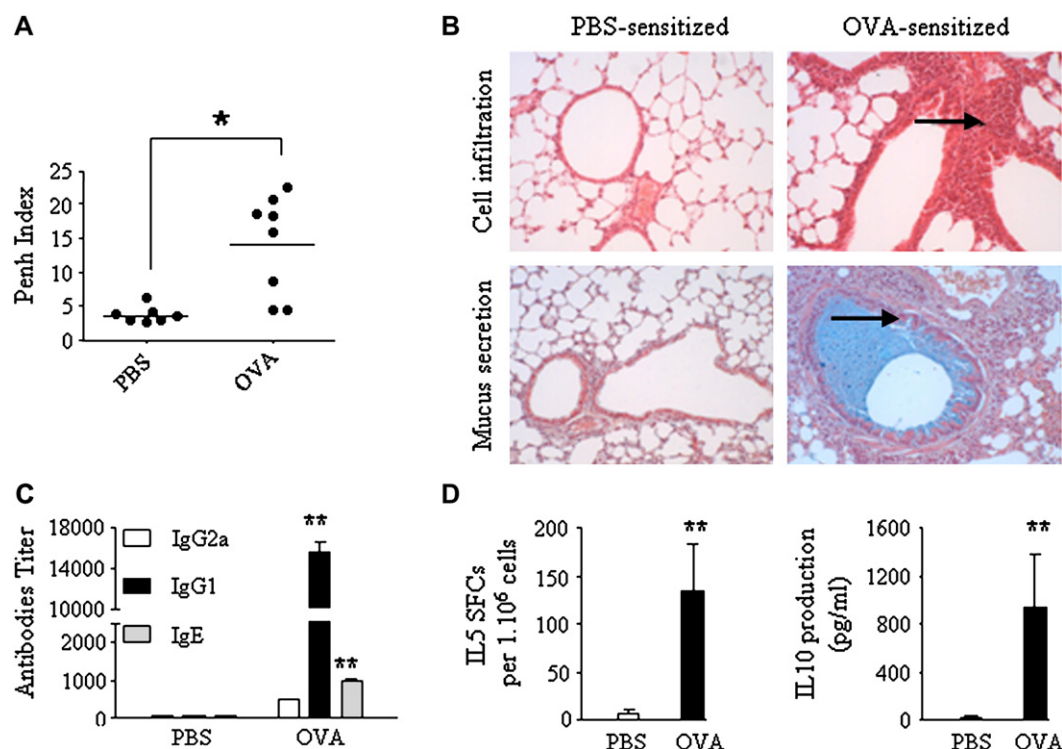


FIG 2. Clinical and immunological assessment of OVA-sensitized mice. Mice sensitized with PBS or OVA were analyzed for AHR, lung inflammation, and humoral and cellular immune responses. **A**, AHR was determined by measuring the Penh index (— = mean). **B**, Representative paraffin lung sections stained with HES (upper panel) or Alcian (lower panel) are shown (200-fold magnification). Arrows in upper and lower panel indicate specific cellular infiltration and mucus secretion, respectively. **C**, OVA-specific IgG_{2a}, IgG₁, and IgE antibody levels were measured in sera by ELISA (means ± SEMs). **D**, IL-5 and IL-10 levels in splenocytes were measured by ELISPOT or ELISA, respectively (means ± SDs). **P* < .05 and ***P* < .02 compared with PBS-treated control mice.

established allergic disease (therapeutic model, Fig 1, C). Mice treated sublingually or by oral gavage (data not shown) with soluble OVA or with OVA adsorbed on a mucoadhesive film (Delstrip-OVA) only slightly reduced AHR compared with PBS-desensitized mice (Fig 4, A), and none of those differences were statistically significant. Interestingly, another formulation based on polymerized carbohydrates (PSC-formulated OVA) exhibited a superior efficacy in terms of reduction of AHR compared with PBS or OVA alone: as shown in Fig 4, B, the Penh index value returned to normal (ie, comparable to healthy nonsensitized mice) in 7 out of 8 mice receiving PSC-formulated OVA. Importantly, a 10-fold lower dose of OVA (50 µg) administered with such a formulation also exhibited a readily detectable significant clinical efficacy in this model (Fig 4, B). This observed decrease of AHR after treatment with PSC-formulated OVA was associated with a significant reduction of bronchial inflammation assessed in terms of both specific cellular infiltration (Fig 5, A) and mucus secretion (Fig 5, B). Eosinophil accumulation was significantly reduced in the lungs of PSC-OVA-treated mice (with a mean number of eosinophils ± SEM of 172 ± 38) compared with PSC control mice (267 ±

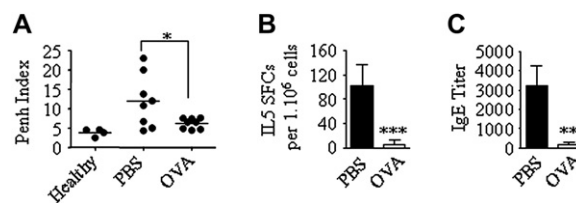


FIG 3. Prophylactic sublingual administration of OVA prevents sensitization. Mice treated sublingually with either PBS or soluble OVA before sensitization were analyzed for AHR, and humoral and cellular immune responses. **A**, AHR was determined by measuring the Penh index (— = mean). The Penh value of healthy mice (nonsensitized) is shown as a control. **B**, IL-5 production in splenocytes was measured by ELISPOT (means ± SDs). **C**, OVA-specific IgE antibody levels were measured by ELISA (means ± SEMs). **P* < .05, ***P* < .02, and ****P* < .001 compared with mice treated with PBS before sensitization.

95). Also, a decrease in perivascular inflammation was noted in the PSC-OVA group (graded ++ vs +++ for all other groups as described in Methods). Importantly, the PSC formulation itself, when administered without OVA, had no effect on either the Penh index (Fig 4, C)

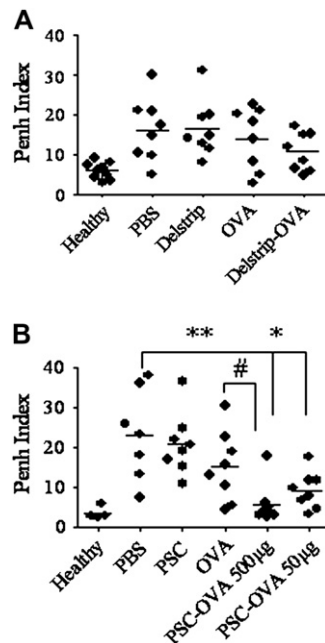


FIG 4. Therapeutic SLIT with formulated OVA reduces established AHR. **A**, AHR was determined by measuring the Penh index in mice desensitized with PBS, soluble OVA, Delstrip alone, Delstrip-OVA (**A**), PSC alone, or PSC-formulated OVA (**B**) by measuring the Penh index (— = mean). * $P < .05$, ** $P < .02$ compared with PBS-desensitized mice. # $P < .05$ compared with OVA-desensitized mice.

or lung inflammation parameters (Fig 5, *A and B*, upper panel).

Therapeutic sublingual administration of formulated OVA reduces IL-5 and IL-10 production in the spleen and elicits lung mucosal IgA responses

We subsequently assessed immune responses in animals successfully treated by SLIT. OVA-specific IL-5 and IL-10 production was significantly reduced in spleen cell cultures from mice treated with PSC-formulated OVA compared with control animals receiving either PBS or PSC alone (Fig 6, *A and B*). The decrease in T_H2 responses was more limited in mice treated with soluble OVA (Fig 6, *A*). We failed to detect IL-10 or TGF- β -producing T cells in cervical, brachial, and axillary LNs (data not shown). No detectable changes in serum OVA-specific IgE or IgG antibodies were observed in any of the groups (data not shown). However, lung mucosal IgA levels were dramatically increased in PSC-formulated OVA-treated mice compared with mice receiving either soluble OVA or PBS (Fig 6, *C*).

T-cell priming occurs in cervical/maxillary lymph nodes after sublingual administration of OVA

To characterize immune responses after sublingual immunization, CFSE-labeled DO11.10 $CD4^+$ T cells were adoptively transferred by intravenous injection into

BALB/c mice before sublingual administration of PBS, OVA, PSC, or PSC-OVA. After 10 days, cervical/maxillary and mesenteric LNs were harvested, and *in vivo* OVA-specific T-cell proliferative responses were analyzed by flow cytometry. As shown in Fig 7, *A*, T-cell proliferation was hardly detectable in cervical/maxillary LNs of mice treated sublingually with PBS or PSC with only 3.6% and 3.4% proliferating T cells, respectively. Sublingual administration of OVA induced a moderate but readily detectable T-cell proliferation in cervical/maxillary LNs, in the range of 9.4%. Strikingly, the use of PSC-OVA dramatically enhanced T-cell proliferation up to 33.9% (Fig 7, *A*). In mesenteric LNs, OVA-specific T-cell proliferation was barely detectable in all groups of mice (Fig 7, *B*), demonstrating that sublingual administration of PSC-OVA (and to a lesser extent of OVA) results in a local priming of the immune system.

DISCUSSION

Several animal studies have demonstrated the efficacy of allergen-specific immunotherapy conducted through the subcutaneous, nasal, or oral routes to establish allergen specific immune tolerance.⁷⁻¹¹ Although SLIT has been in clinical use in human beings for more than 15 years, very few experiments have been conducted in animal models.¹⁷⁻¹⁹ Most existing animal models are performed in a prophylactic setting (ie, SLIT conducted before or in parallel with allergen challenge), whereas in human beings, SLIT is always used therapeutically. Also, a major limitation has been to control the dose and duration of administration when using the sublingual route in mice. In this context, we developed a therapeutic SLIT model in BALB/c mice with OVA-induced asthma, in which the antigen is administered with a mucoadhesive formulation.

When performed before sensitization, SLIT with soluble OVA prevents AHR and T_H2 immune responses, consistent with other studies conducted via the oral or sublingual route in rats and mice.^{10,17,19} In contrast, only therapeutic sublingual administration of formulated but not soluble OVA reduces both established AHR and bronchial inflammation. Similarly, Sun et al¹⁸ have recently reported that adjuvanted OVA (ie, conjugated to the cholera toxin B subunit) but not OVA alone is clinically efficient when used sublingually in mice. In our model, clinical benefit was associated with both a decrease in OVA-specific IL-5 and IL-10 production by splenocytes, and IgA secretion in the lungs. In contrast, no effect on established IgE or IgG serum responses was detected during therapeutic SLIT. Interestingly, those biological changes recapitulate the ones observed in human beings during SLIT, including a downregulation of T_H2 cytokine production²⁰ as well as an upregulation of IgA responses.^{21,22}

In current SLIT protocols, the allergen extract administered as a solution without any adjuvant or formulation is held under the tongue for 1 to 2 minutes before being

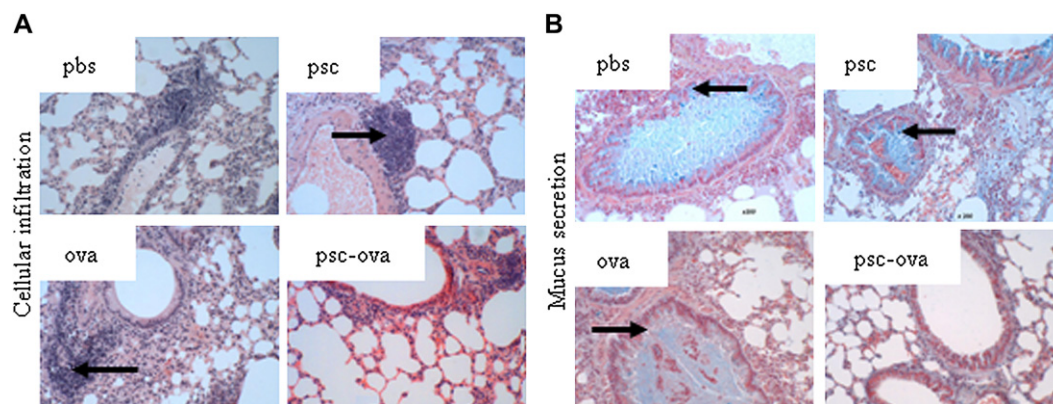


FIG 5. Therapeutic SLIT with formulated OVA reduces bronchial inflammation. Representative paraffin lung sections stained with HES (A) or Alcian (B) from mice desensitized with either PBS, soluble OVA, PSC alone, or PSC-formulated OVA are shown (200-fold magnification). Arrows indicate cellular infiltration (A) or mucus secretion (B).

swallowed. Under those conditions, no significant plas-matic absorption of allergens occurs through the sublin-gual mucosa and only a small fraction (ie, < 10%) remains in contact for as long as 20 hours with the mucosa.^{23,24} Our results suggest that mucoadhesive formulations will allow reduction of the allergen dose by at least 10-fold while sustaining efficacy, presumably by increasing the amount of allergen in contact with the mucosa and/or the duration of contact. The better efficacy of PSC-OVA relative to soluble OVA in reducing AHR and inflammation demonstrates that improving allergen contact with the sublingual mucosa enhances tolerance induction, presumably by facilitating allergen capture by oral Langerhans-like dendritic cells.²⁵ This hypothesis is consistent with the clear enhancement of T-lymphocyte priming in cervical lymph nodes with PSC-OVA compared with soluble OVA.

The availability of a murine SLIT model will allow investigation in detail of immune mechanisms associated with SLIT-induced improvement of the respiratory function. Consistent with the decrease in T_H2 allergic responses seen in our murine model, high-dose sublingual immunotherapy could induce anergy, T-cell deletion, or stimulation of regulatory T cells. Indeed, regulatory responses are known to downregulate allergic responses through the secretion of cytokines such as TGF- β or IL-10, or via cell-cell contact.^{26,27} Although it has been established that subcutaneous immunotherapy induces such regulatory T cells in human beings,^{26,28-30} there is only preliminary evidence that this is the case as well for SLIT in human beings.³¹⁻³⁴ In mice, it has been recently shown that SLIT with OVA fused to the cholera toxin B subunit induces Foxp3⁺CD4⁺CD25⁺ regulatory T cells in cervical lymph nodes.¹⁸ In our murine SLIT model, we failed to detect the induction of antigen-specific IL-10 or TGF- β -producing T cells in the spleen or in draining lymph nodes of cured animals (data not shown). Interestingly, however, preliminary experiments suggest that suppression of AHR can be transferred to OVA-sensitized animals with splenic

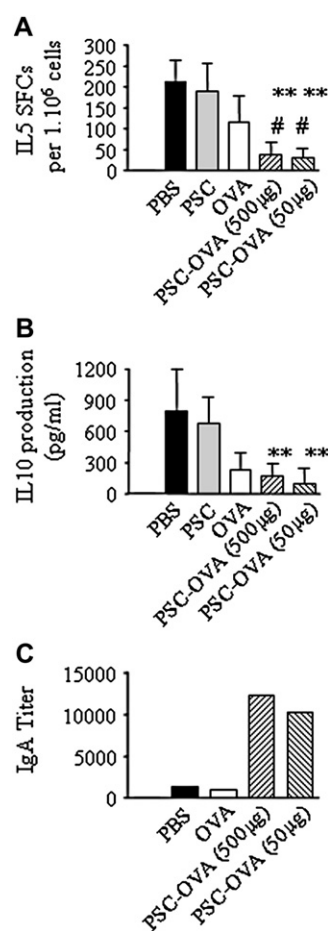


FIG 6. Therapeutic SLIT with formulated OVA reduces OVA-specific T_H2 responses in splenocytes and induces lung mucosal IgA production. Mice were desensitized with PBS, soluble OVA, PSC alone, or PSC-formulated OVA. IL-5 (A) and IL-10 (B) levels in splenocytes were measured by ELISPOT or ELISA, respectively (means $\pm$ SDs). ** P < .02 compared with PBS-desensitized mice. # P < .05 compared with OVA-desensitized mice. C, OVA-specific IgA antibody levels were measured in lungs by ELISA.

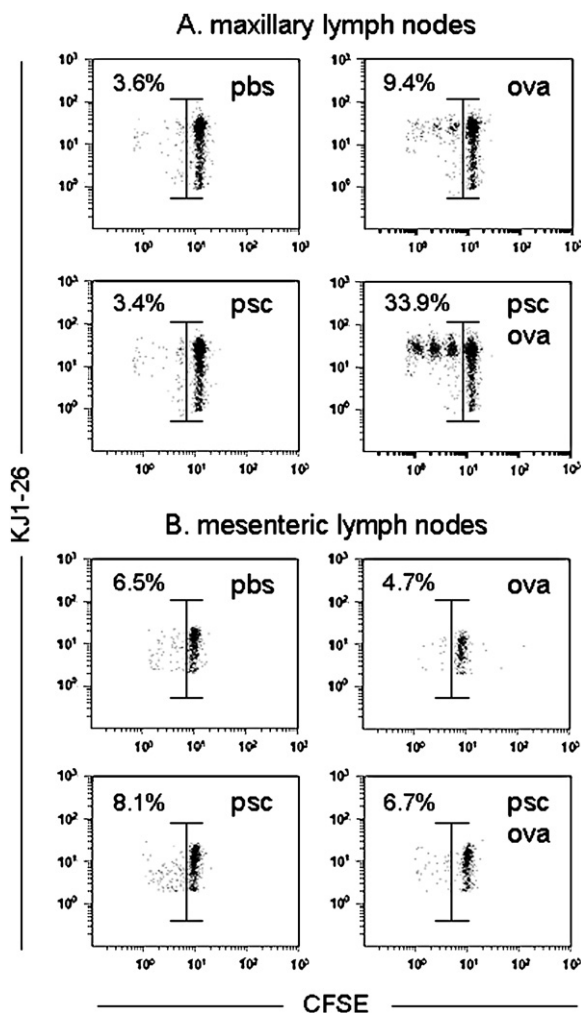


FIG 7. T-cell priming in cervical/maxillary LNs after sublingual administration of PSC-OVA. Purified CFSE-labeled DO11.10 CD4⁺ T cells were adoptively transferred into BALB/c mice at day 0. Twenty-four hours later, mice were treated by the sublingual route with soluble OVA or PSC-OVA. Control animals were treated with either sterile PBS or the PSC formulation alone. At day 10, cervical/maxillary (A) and mesenteric (B) LNs were removed. Proliferating cells were detected by FACS as cells with a decreased fluorescence. Data are representative of 3 independent experiments.

T cells from mice treated with PSC-formulated OVA (data not shown). That PSC-OVA specifically induces a strong proliferation of OVA-specific T cells in draining lymph nodes is also consistent with the hypothesis that tolerance induction via the sublingual route involves antigen-specific CD4⁺ T cells. The pattern of differentiation of such proliferating T cells, possibly toward a regulatory T-cell phenotype, is currently under investigation.

Altogether, our results unambiguously document in a therapeutic murine model the value of mucoadhesive formulations to enhance SLIT efficacy. This murine model will be useful to evaluate simplified administration schemes and to select adjuvants or formulations to incorporate in second-generation sublingual vaccines.

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TABLE E1. Overview of group characteristics for therapeutic SLIT

Groups of mice	Sensitization* (number of mice)	Desensitization† (number of mice)	AHR (number of mice)	Lung histology§ (number of mice)	Clinical and immunologic readouts‡		
					Humoral and mucosal responses (number of mice)		Cellular responses (number of mice)
					Blood	Lung	
Healthy	NT (8)	NT (8)	(8)	ND	ND	ND	ND
PBS	OVA (8)	PBS (8)	(8)	(8)	(8)	(Pool of 8)	(8)
OVA	OVA (8)	OVA (8)	(8)	(8)	(8)	(Pool of 8)	(8)
Formulation	OVA (8)	Formulation (8)	(8)	(8)	(8)	(Pool of 8)	(8)
Formulation + OVA	OVA (8)	Formulation + OVA (8)	(8)	(8)	(8)	(Pool of 8)	(8)

ND, Not determined; NT, not treated.

*Mice were sensitized by 2 intraperitoneal injections of OVA and alum at 14 days intervals. This was followed by aerosol challenges with OVA on 4 consecutive days.

†Mice were weekly desensitized (2 sublingual administrations) with PBS, OVA solution, formulation alone, or formulation + OVA for 2 months.

‡Readouts were AHR (Penh value), lung histology, humoral responses (OVA-specific IgG₁, IgG_{2a}, IgE) assessed by ELISA, mucosal response (OVA-specific IgA) measured by ELISA, and cellular responses analyzed by ELISA and ELISPOT.

§After mice were killed, lungs were fixed and embedded in paraffin wax. Lung sections were stained with HES and Alcian blue. Thereafter, perivascular inflammation and eosinophil infiltration were scored.