

Sublingual immunotherapy in sensitized mice

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Background: Many studies have demonstrated immunologic changes induced by sublingual immunotherapy (SLIT), but the definitive mechanism of action needs further investigation.

Objective: To study the immunologic response induced by SLIT in sensitized mice.

Methods: Timothy grass (*Phleum pratense*)–sensitized mice received SLIT for 2, 4, or 6 weeks at 3 different concentrations, including a buffer control. Serum samples and washes of the lungs (bronchoalveolar lavage [BAL]) and the nasal passages (nasal lavage [NAL]) were analyzed for allergen-specific antibodies. T cells were isolated from the spleen and cervical lymph nodes for the analysis of proliferation and cytokine production.

Results: Sublingual immunotherapy in sensitized mice resulted in a 30-fold increase in antigen specific IgA levels in BAL and NAL fluid compared with buffer-treated mice, whereas antigen specific IgE was undetectable in BAL and NAL fluid in animals treated with SLIT. Furthermore, IgA levels were proportional to the dose and duration of SLIT. Levels of specific IgA in serum correlated with levels in BAL and NAL fluid. Serum IgA levels were proportional to the duration of allergen exposure to the oral mucosa. Conversely, no changes in serum levels of IgE and IgG were induced by SLIT. Proliferation of T cells was increased in mice treated with SLIT compared with nontreated mice.

Conclusion: High levels of IgA in serum and in BAL and NAL fluid of mice treated with SLIT demonstrate that SLIT induces a mucosal, nonallergic response in sensitized mice.

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INTRODUCTION

Several double-blind, placebo-controlled studies^{1–5} have recently demonstrated that sublingual immunotherapy (SLIT) is a clinically effective treatment for pollen- and house dust mite-induced rhinitis and asthma. Numerous studies^{1–5} demonstrate that levels of allergen specific IgE are either increased or remain unaffected by SLIT. Furthermore, in most studies,^{1,3,6–8} levels of allergen specific IgG4, which is thought to reflect a modulation in the allergic T_H2 response, are increased in patients actively treated with SLIT. A recent study,⁹ however, indicates that SLIT in children does not induce changes in specific IgG4 levels but down-regulates the allergen specific IgE response and stimulates the production of IgA antibodies. In naïve rats, SLIT selectively down-regulates specific IgE responses,¹⁰ and in naïve mice, SLIT with lipopeptides resulted in the induction of interferon γ (IFN- γ)–producing T cells and of IgG2a antibody production.¹¹

Apart from modulating the antibody response, SLIT has been demonstrated to lead to a decrease in infiltrating nasal effector cells and serum eosinophil cationic protein levels,^{12,13} indicating reduced inflammation in SLIT-treated patients. Also, the expression of intercellular adhesion molecule 1 on nasal epithelium has been reported to decrease in patients treated with SLIT.¹³ Although Fanta et al⁶ reported that the in vitro proliferation of allergen-specific spleen cells is reduced in patients treated with SLIT, this is not associated with a

change in the cytokine profile. In line with this, SLIT does not seem to affect the level of interleukin 12 (IL-12) messenger RNA–positive cells in biopsy samples of sublingual mucosa.³ However, Ippoliti et al^{12,13} demonstrated that the serum concentration of IL-13 is decreased after 6 months of SLIT, suggesting that SLIT might operate by modulating the T_H2-biased allergic immune response. Despite the many trials demonstrating the immunomodulatory effect of SLIT, the mechanisms by which this is accomplished still remain elusive. To address the immunologic mechanisms of SLIT, we developed an animal model. The model demonstrates substantial changes in serum IgA levels and in the mucosal IgA response on SLIT with an extract of Timothy grass (*Phleum pratense*).

METHODS

Animals

Female 6- to 10-week-old Balb/cJ mice were bred in-house and were maintained on a defined diet that did not contain components that cross-react with antisera to *P pratense* extract. The mice were housed in a specific pathogen-free environment under a 12-hour light, 12-hour dark cycle. All experiments described herein were conducted in accordance with Danish legislation.

Experimental Design

Each experimental group consisted of 4 to 10 animals. The mice were sensitized by means of 3 intraperitoneal injections of alum-adsorbed *P pratense* extract (ALK-Abelló) (Fig 1). Each injection contained 5,000 standardized quality (SQ) units of *P pratense* extract, corresponding to approximately 1 μ g of the major allergen Phl p5 or 1.85 μ g of total protein. After sensitization, serum samples were collected and aller-

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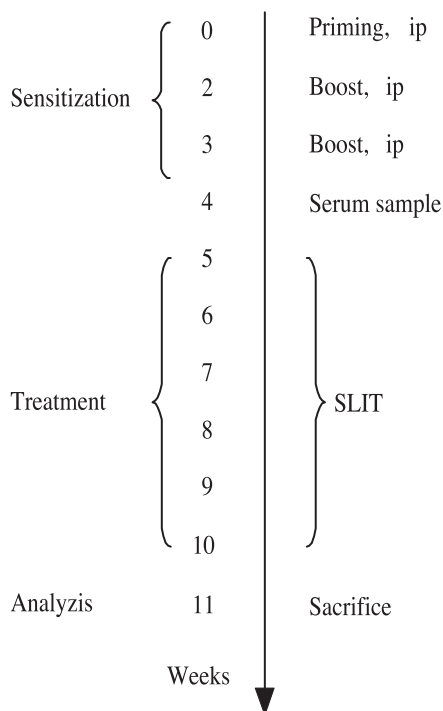


Figure 1. Experimental design and sampling. Week 0 is defined as the start of the experiment. The mice were sensitized by intraperitoneal (ip) injections before sublingual immunotherapy (SLIT). Blood samples were collected at indicated times, and serum, bronchoalveolar lavage fluid, and nasal lavage fluid samples were collected when the animals were humanely killed. In addition, the spleen and cervical lymph nodes were removed for in vitro proliferation and cytokine assays.

gen specific IgG1, IgG2a, and IgE levels were measured. Based on these antibody measurements, the mice were stratified into treatment groups. Sublingual immunotherapy was performed by holding the scruff of the mouse and carefully applying 5 μ L of allergen solution under the tongue. The mouse was held by the scruff for 20 seconds to prevent the animal from immediately swallowing the allergen solution. Unless specified, the mice received SLIT for 6 weeks (5 d/wk). The distribution of sublingually applied solutions was investigated using a 0.5% Evans blue solution. The mice were humanely killed 5 minutes after administration, and the distribution of the dye in the sublingual tissue, esophagus, stomach, and muscle (negative control) was evaluated by means of visual inspection and extraction of tissue in an acetone–0.5% sodium sulfate solution, followed by measurement of absorption at 620 nm. Blood samples were collected at relevant times, and washes of the lungs (bronchoalveolar lavage [BAL]) and nasal passages (nasal lavage [NAL]) were collected at the time of humane killing. The spleen and cervical lymph nodes were collected for in vitro analysis of *P pratense*–specific proliferation and cytokine secretion. Serum samples were analyzed for allergen specific IgE, IgA, IgG1, and IgG2a. Specific IgA and IgE levels were measured in serum, BAL fluid, and NAL fluid.

Measurement of Specific Antibodies

Allergen specific IgG1 and IgG2a antibodies were measured in an indirect enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent plates were coated with *P pratense* extract (10 μ g/mL in phosphate-buffered saline, pH 7.2). After the addition of diluted sera, the antibodies were detected using biotinylated goat anti–mouse IgG1 or biotinylated rat anti–mouse IgG2a antibodies (Southern Biotech, Birmingham, AL, and BD Biosciences Pharmingen, Franklin Lakes, NJ, respectively). Streptavidin-peroxidase (Dako, Glostrup, Denmark) was added, and the plates were developed using 3,3',5,5'-tetramethylbenzidine (TMB; Kem-En-Tec Diagnostics, Taastrup, Denmark). Absorbances were read at 450 nm.

Allergen specific IgE and IgA antibodies were measured in a Magic Lite assay (Ciba Corning Diagnostic Corp, Medfield, Mass). Briefly, serum, BAL fluid, or NAL fluid was diluted in wash buffer (0.06% gelatin and 0.1% Triton X-100 in phosphate-buffered saline, pH 6.65) and incubated with magnetic particles coupled to goat anti–mouse IgA or rat anti–mouse IgE (Indicia Biotechnology, Oullins, France). After washing, allergen-specific antibodies were detected by means of biotinylated *P pratense* extract and streptavidin conjugated to acridinium ester. Visualization was performed in a luminometer, with automated injection of peroxide and hydrochloric acid. The technical characteristics of the assay are described elsewhere.¹⁴ Serum IgA levels specific for major allergens of *P pratense* extract, ie, Phl p1, p2/3, p5, and p6, were measured using the ADVIA Centaur platform (Siemens Medical Solutions Diagnostics, Tarrytown, NJ).¹⁵ Briefly, goat anti–mouse IgA (Southern Biotech) covalently coupled to paramagnetic particles was used to capture serum IgA. Bound solid-phase IgA was allowed to react with liquid-phase purified biotinylated major allergens, which were detected as chemiluminescence using acridinium ester–labeled streptavidin.

T-Cell Proliferation and Cytokine Production

Spleens were teased into single-cell suspension and washed 3 times in RPMI 1640 medium (Cambrex Bio Science, Baltimore, MD). Cells were counted and adjusted to 1.67×10^6 cells/mL in RPMI 1640 containing 50 μ g/mL of gentamycin (Invitrogen Corp, Carlsbad, CA), 1% Nutridoma (Roche Applied Science, Indianapolis, IN), and 1.5-mmol/L monothio-glycerol (Sigma-Aldrich Corp, St Louis, MO). A total of 3×10^5 cells was added to each well of a 96-well flat-bottomed culture plate (Nalge Nunc International, Rochester, NY), and the cells were stimulated by 0, 10, and 40 μ g/mL of Phl p extract. The cells were cultured for 6 days at 37°C in 5% carbon dioxide. Proliferation was measured by adding 0.5 μ Ci of ³H-thymidine to each well for the last 18 hours of the culture period, followed by harvesting the cells on a 96-well plate harvester (Tomtec, Hamden, CT) and counting the incorporated radiolabel using a scintillation counter (Wallac model 1450; PerkinElmer, Waltham, MA). For cytokine production the cells were adjusted to 3×10^6 cells/mL and were

added to each well together with medium or 40 $\mu\text{g/mL}$ of Phl p extract. Supernatants were harvested at day 5 and were analyzed using a CBA kit (BD Biosciences Pharmingen).

Statistical Analysis

All statistical calculations were performed using a software program (GraphPad Prism version 4.02; GraphPad Software Inc, San Diego, CA). Differences were considered statistically significant at $P < .05$.

RESULTS

Antibody Response After SLIT in Sensitized Mice

We first investigated whether SLIT in mice induces changes in the serum antibody levels. Owing to sensitization of the mice by means of 3 intraperitoneal injections, levels of IgG and IgE were elevated compared with those in nonsensitized, naïve animals (data not shown). Groups of mice received daily doses of 0 (buffer control), 5, 25, or 125 kSQ units of *P pratense* extract sublingually. The daily doses are equivalent to the doses administered in human clinical trials. In these trials, the daily doses ranged from 2.5 to 75 kSQ.^{16,17} The efficacy of SLIT depends on the duration of preseasonal treatment. For example, better symptom and medication scores were observed for 16 vs 8 weeks of preseasonal treatment in human clinical trials.^{16,17} For practical reasons, the mice in this study were treated for 2, 4, or 6 weeks. Six weeks of SLIT in sensitized mice did not generate increased serum levels of specific IgE, IgG1, and IgG2a compared with sensitized mice receiving buffer sublingually (Fig 2). Furthermore, compared with buffer-treated mice, no down-regulation of the antibody response was observed, suggesting that SLIT in sensitized mice did not induce active suppression of the immune response. Likewise, SLIT of sensitized mice for 2 or 4 weeks did not generate altered serum IgG or IgE levels (data not shown).

Whereas SLIT did not modulate serum levels of IgG and IgE, serum and mucosal IgA levels were increased in SLIT-treated mice compared with buffer-treated mice. The specific mucosal IgA levels were proportional to the duration of SLIT and to the dose of *P pratense* extract administered (Fig 3). Comparing median values for IgA in NAL fluid, SLIT for 2 and 6 weeks at a daily dose of 25 kSQ increased 3.4-fold. Thus, in this experimental design, an additional 4 weeks of SLIT generated almost a 4-fold increase in IgA levels in NAL fluid. Furthermore, IgA levels increased when increasing the concentration of allergen extract administered via the sublingual route. Specific IgA levels in NAL fluid increased 8.7-fold comparing untreated mice (0 kSQ) with mice receiving 5 kSQ of *P pratense* extract daily for 6 weeks. Comparing untreated mice with mice receiving an extract dose of 25 kSQ daily for 6 weeks, IgA levels in NAL fluid increased 30-fold. Comparing 6 weeks of SLIT at doses of 25 and 125 kSQ, there seemed to be a tendency toward a plateau or maximum at the intermediate dose of 25 kSQ. Because the higher dose did not seem to increase IgA production, a treatment regimen of 25 kSQ for 6 weeks was adapted for all further experi-

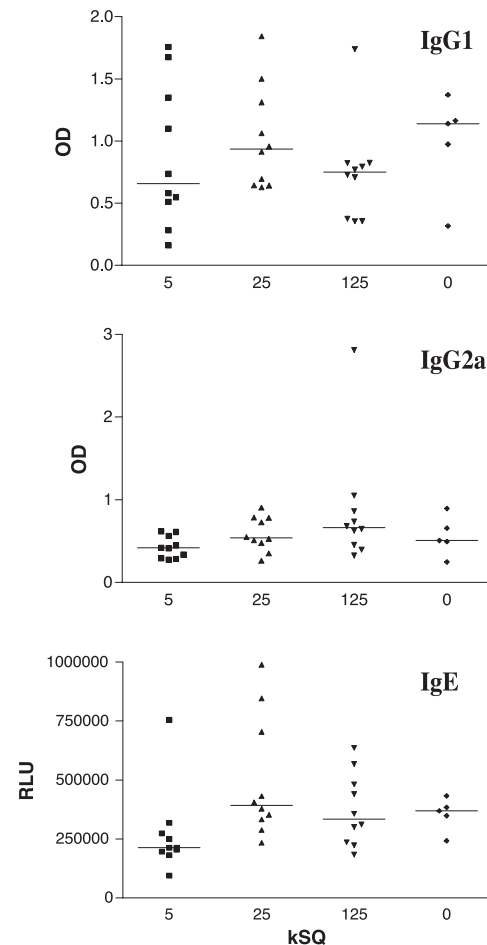


Figure 2. Serum levels of specific IgG1, IgG2a, and IgE in sensitized mice treated with sublingual immunotherapy for 6 weeks. Each group of mice received daily sublingual immunotherapy doses of 0 (buffer control), 5, 25, or 125 kSQ. Serum dilution for IgG1 was 1:250,000; IgG2a, 1:2,000; and IgE, 1:10. OD indicates optical density at 450 nm; RLU, relative light units. The points represent measurements of individual mice; horizontal lines, medians.

ments. Antigen specific IgA levels in BAL fluid and serum revealed a similar response pattern as that observed with the levels in NAL fluid (data not shown).

The BAL and NAL fluids of SLIT- and buffer-treated mice were also analyzed for the presence of *P pratense* specific IgE antibodies. As opposed to murine asthma disease models, we did not find IgE antibodies in BAL or NAL fluid, probably owing to the lack of pulmonary inflammation in the SLIT-treated mice and the buffer-treated mice (data not shown).

Specificity Toward Major Allergens Phl p1, p2/3, p5, and p6

To dissect the immunogenicity of individual *P pratense* allergens, when whole pollen extract is applied to oral mucosa, we analyzed serum IgA reactivity to purified, biotinylated Phl

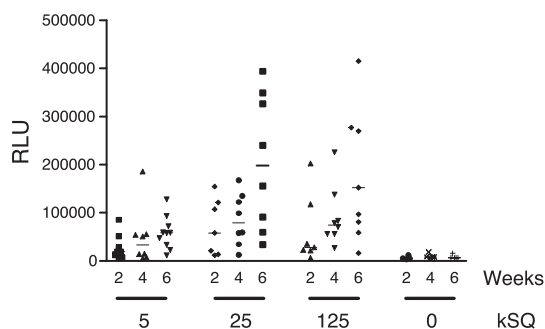


Figure 3. Levels of IgA in nasal lavage fluid of sublingual immunotherapy-treated sensitized mice. The mice were allocated to groups receiving daily sublingual immunotherapy doses of 0 (control), 5, 25, or 125 kSQ for 2, 4, or 6 weeks. RLU indicates relative light units. The points represent measurements of individual mice; horizontal lines, medians.

p1, p2/3, p5, and p6. As shown in Figure 4, Phl p5 and p6 generated strong IgA responses in SLIT-treated animals. All SLIT-treated mice generated Phl p5 specific IgA antibodies, whereas Phl p6 specific antibodies were observed in 6 of 10 animals. Specific IgA antibodies against Phl p1 and p2/3 were generated, albeit at lower levels. Comparing the highest relative light unit value for mice not receiving SLIT with values for actively treated mice, specific antibodies for Phl p1 and p2/3 were seen in 3 and 5 of 10 animals, respectively.

SLIT May Be Absorbed Through the Oral Mucosa

We next investigated whether serum IgA levels correlate with the period of contact between the *P pratense* extract and the oral mucosa. To prevent the animals from swallowing the allergen solution immediately, mice were held by the scruff after the application of SLIT under the tongue. Using Evans blue as a tracer dye, we demonstrated that dye was present in the stomach to an equal extent in mice that were allowed to swallow the allergen solution immediately and in mice that were held by the scruff for 20 seconds (data not shown). This finding indicates that this model of SLIT emulates the so-called sublingual-swallow method, which is used in most clinical studies.¹⁸ To determine the importance of the duration of allergen contact with the oral mucosa, the mice were held for 0, 20, or 40 seconds or were administered the same dose of allergen by gastric gavage. As shown in Figure 5, serum IgA levels were 4-fold increased when the allergen solution was kept in contact with the oral mucosa for 20 seconds compared with in animals that were allowed to swallow the allergen solution immediately. However, if the duration of contact between allergen and the oral mucosa was increased to 40 seconds, no further increase in serum IgA levels was observed compared with levels at 20 seconds of contact. If the animals received the same dose of allergen by gastrointestinal instillation, IgA levels were comparable with the levels observed for animals that were allowed to swallow

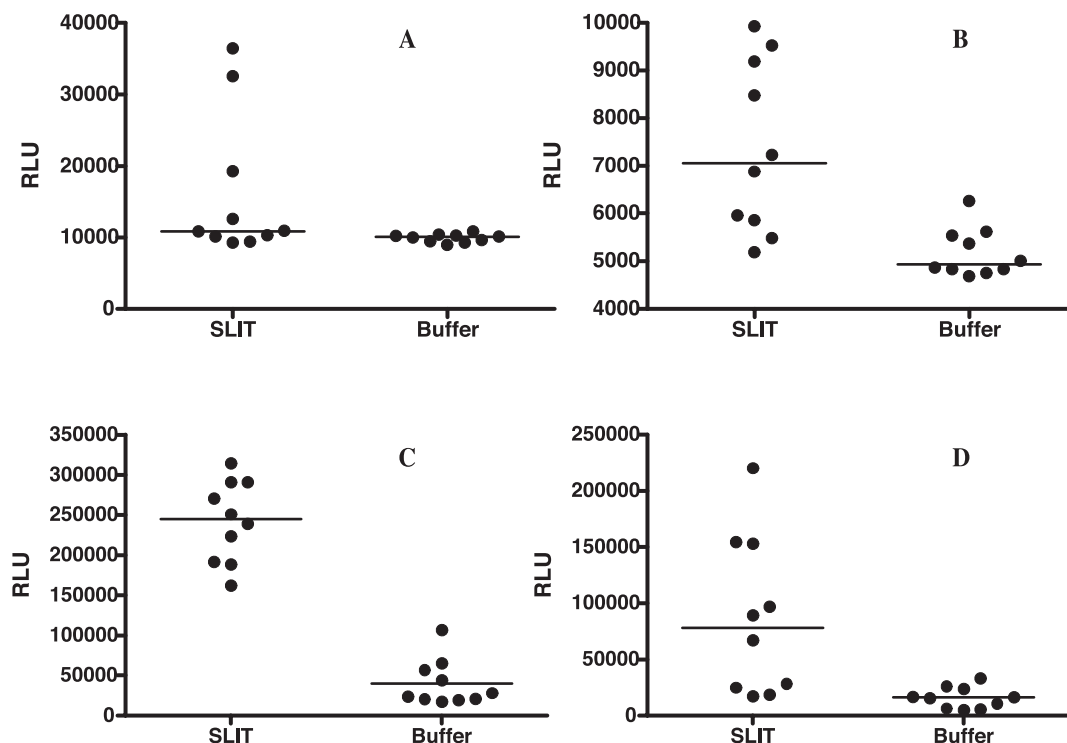


Figure 4. Serum IgA antibodies specific for major *Phleum pratense* allergens: Phl p1 ($P = .04$) (A), Phl p2/3 ($P = .001$) (B), Phl p5 ($P < .0001$) (C), and Phl p6 ($P = .001$) (D). Sublingual immunotherapy (SLIT) doses were either 0 (buffer) or 25 kSQ. RLU indicates relative light units. The points represent measurements of individual mice; horizontal lines, medians. Statistical test: Mann-Whitney U test.

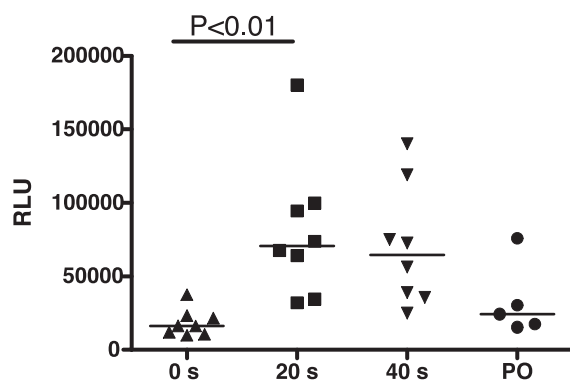


Figure 5. Specific serum IgA levels correlate with the duration of contact between allergen and the oral mucosa. Sublingual immunotherapy doses were 25 kSQ. The mice were held for 0, 20, or 40 seconds or were given extract by oral gavage (PO). RLU indicates relative light units. The points represent measurements of individual mice; horizontal lines, medians. Statistical test: Mann-Whitney *U* test.

immediately (0 seconds). This experiment indicates a significant role for the oral mucosa in SLIT. Furthermore, it suggests that the oral mucosa assimilates or actively transports the allergen solution across the epithelium for processing in the context of the immune system.

T-Cell Response After SLIT of Sensitized Mice

To evaluate the local and systemic T-cell responses, the allergen-specific *in vitro* response of T cells isolated from the cervical lymph nodes and the spleen was analyzed. As shown in Figure 6, the proliferative response was increased for systemic and local T cells in mice treated with SLIT compared with buffer-treated animals, indicating an activating effect of SLIT on the T-cell response after stimulation with antigen. Cell supernatants of T-cell cultures were analyzed for cytokine production. T cells from cervical lymph nodes of SLIT- and buffer-treated mice produce similar quantities of IFN- γ , IL-4, and IL-10. For SLIT-treated mice, there was a tendency toward lower levels of IL-5 compared with buffer-treated mice; however, these differences were not significant. In cultures of T cells isolated from the spleen, comparable amounts of IFN- γ , IL-4, and IL-5 were observed for both groups of mice. Although not statistically significant ($P = .06$), IL-10 seemed to be up-regulated for SLIT-treated mice in cultures of T cells isolated from the spleen.

DISCUSSION

In this study, SLIT of sensitized mice does not seem to induce active tolerance as measured by reduction of serum levels of specific antibodies or T-cell proliferative activity. On the contrary, high levels of IgA in serum, BAL fluid, and NAL

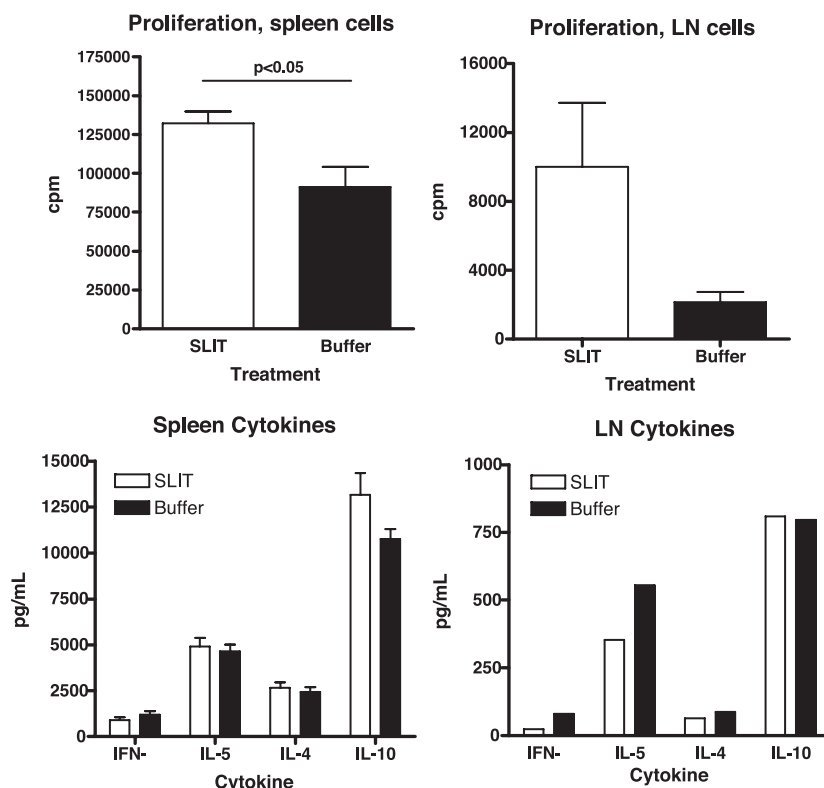


Figure 6. Proliferation and cytokine production of T cells from the spleen and cervical lymph nodes (LNs) of mice treated with sublingual immunotherapy (SLIT) or buffer for 6 weeks. Each column represents the mean of 8 individual mice; error bars, SEM. No error bars indicates measurements for pooled cells from SLIT- or buffer-treated animals. IFN indicates interferon; IL-4, interleukin 4. Statistical test: *t* test.

fluid of SLIT-treated mice suggest induction of a mucosal immune response.

Furthermore, we showed a positive association between the levels of serum IgA antibodies and the duration of contact between the allergen and the oral mucosa. The serum IgA levels in mice not allowed to swallow the allergen solution for 20 seconds were 4-fold higher than those in mice allowed to swallow immediately. Levels of IgA in mice receiving the allergen solution by gastrointestinal instillation, thus bypassing the sublingual epithelium, were comparable with levels in mice swallowing immediately. This is in agreement with the early study by Holt et al.¹⁰ Although studying SLIT in non-sensitized rats, Holt et al.¹⁰ did not observe down-regulation of the IgE response after direct gastrointestinal instillation of the allergen, suggesting that allergens are indeed taken up locally after sublingual administration. Other studies^{19,20} indicate absorption and antigen presentation via dendritic cells in murine buccal epithelium by means of immunochemical analysis and dosing with radiolabeled allergen. These observations suggest that nonencapsulated protein allergens are absorbed and processed by cells in the oral epithelium and that nonencapsulated *P pratense* allergens are degraded in the gastrointestinal tract.²¹

The immunobiologic profile of grass pollen allergens is well described for humans and mice. In humans, Phl p1, p4, p5, and p6 are considered major allergens, whereas the prevalence of IgE antibody reactivity to Phl p2/3 is in the range of 40% to 60%, depending on the study population or patient selection criteria.²² When Balb/c mice are immunized with an extract of *P pratense*, Western immunoblotting reveals that the IgE reactivity is similar to that found in allergic patients, except for Phl p1.²³ This finding cannot be due to lack of immunogenicity, because Phl p1 specific IgE antibodies are generated if mice are immunized with purified Phl p1. However, this suggests that Phl p1 is prevented from mounting an IgE response when mice are sensitized via injection by whole pollen extract.²³ We showed that sublingual administration of a natural *P pratense* extract in sensitized mice elicits an immune response characterized by production of IgA antibodies recognizing 4 of 5 major allergens of *P pratense*, ie, Phl p1, p2/3, p5, and p6. This indicates that at least these major allergens could be taken up and processed in the context of the mucosal immune system. When comparing the number of animals generating specific IgA antibodies to individual major allergens, Phl p2/3, p5, and p6 are most potent compared with mice treated sublingually with buffer. However, Phl p1 also generates specific IgA antibodies in 3 of 10 mice receiving SLIT with whole extract of *P pratense*. Although 65% to 100% of Timothy grass-allergic patients mount an IgE response to this particular major allergen,^{24–26} immunization of Balb/c mice with whole extract of *P pratense* did not mount an IgE response against Phl p1.²³ Because allergic patients are sensitized via the respiratory system, these findings indicate that to elicit an antibody response to Phl p1, this allergen may have to be introduced via the mucosal route.

We showed that SLIT in sensitized mice induces production of secretory antigen specific IgA. It is unknown whether production of allergen specific IgA in the airway mucosa is beneficial to allergic patients. It may be argued that induction of IgA promotes clearance of allergens. Yet, production of allergen specific IgA in the airways may be problematic because the most potent stimulator of eosinophil degranulation is IgA, and increased levels of IgA have been detected in induced sputum of allergic asthmatic patients.^{27,28} A third possibility is that allergen specific IgA is merely an epiphenomenon related to activation of the mucosal immune system and plays a nonsignificant role in the pathogenesis of allergy and asthma. To address these issues, the physiologic role of mucosal allergen specific IgA is presently under investigation by the use of animal models of allergic diseases.

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