Response to sublingual immunotherapy with grass pollen extract: Monotherapy versus combination in a multiallergen extract

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Background: To date, there have been no randomized, double-blind studies showing the effectiveness of sublingual immunotherapy with multiple allergens.

Objective: The purpose of this study was to examine whether the efficacy of sublingual immunotherapy (SLIT) with standardized timothy extract was reduced by combination with other allergen extracts.

Methods: A single-center, randomized, double-blind, placebo-controlled trial with SLIT was conducted. After an observational grass season, SLIT was administered for 10 months to 54 patients randomized to 1 of 3 treatment arms: placebo, timothy extract (19 μg Phl p 5 daily) as monotherapy, or the same dose of timothy extract plus 9 additional pollen extracts. Symptom and medication scores were collected and titrated nasal challenges, titrated skin prick tests, specific IgE, IgG_4 and cytokines release by timothy-stimulated lymphocyte proliferation were performed.

Results: Perhaps because of a very low grass pollen season in 2008, there were no significant differences in medication or symptom scores in either treatment group compared with placebo. Compared with placebo, in the timothy monotherapy group, thresholds for titrated nasal challenge and skin prick tests ($P = .03$ and $P = .001$, respectively), and serum-specific IgG_4 levels ($P = .005$) significantly increased, and IFN-γ levels decreased ($P = .02$), whereas in the multiallergen group, there was significant improvement only in the titrated skin prick tests ($P = .04$) which was less than in the monotherapy group. There were no significant differences between the 2 active groups in any outcome measure, and both active groups experienced more adverse events than placebo. There were no systemic reactions.

Conclusion: Improvement in multiple relevant outcomes strongly suggests that SLIT with timothy extract alone was effective; however, the results for symptom and medication scores were not significant. The differences between multiple allergen SLIT and placebo only in skin sensitivity to timothy suggest a reduction in SLIT efficacy in this group. However, further studies are required to confirm these observations.
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Key words: *Sublingual immunotherapy, timothy grass extract, multiple allergen immunotherapy, Phl p 5, cytokine, skin prick tests, nasal challenge, allergen-specific IgE, allergen-specific IgG_4*

Allergic rhinitis continues to be a common problem throughout the world.[1] Allergen immunotherapy is the only treatment available that may alter the course of allergic disease and is an effective form of treatment for both allergic rhinitis and asthma.[2] Subcutaneous immunotherapy is of proven efficacy, but because of safety issues and convenience, alternative methods of administration, including sublingual immunotherapy (SLIT), have been studied. SLIT has been shown to be effective in reducing symptom and medication scores in a recent meta-analysis by the Cochrane Review.[3] However, with the exception of 1 open study of birch and grass extracts, all studies have involved treatment with a single allergen.[4]

One of the potential limitations of SLIT in the United States is that patients tend to have and be treated for multiple clinically relevant sensitivities, whereas in European studies, only 1 predominating sensitivity is usually treated. A US immunotherapy prescription has an average of 10 different extracts.[5] Although multiple allergen immunotherapy has been shown to be effective when administered subcutaneously in some[6-8] but not all[9] studies, to date there have been no randomized, double-blind studies involving SLIT with multiple allergens. In addition, the safety of multiallergen therapy is not known. Recently, 2 cases of anaphylaxis to SLIT associated with multiallergen treatment have been reported in the literature, although in each case, there were reasons for possible increased risk for a reaction.[10,11] There is some suggestion that there may be limited absorptive capacity in the sublingual mucosa that may make treatment with multiallergen SLIT less effective.[12] Therefore, we conducted a study to determine whether monotherapy SLIT was effective in the treatment of predominantly polysensitized patients with grass allergy using a standardized US extract of timothy pollen, and we included a second treatment group receiving the same dose of timothy extracts but now combined with 9 unrelated pollen extracts in the same treatment vaccine. The purpose of the second treatment group was to determine whether the multiallergen mixture was also effective in SLIT.

METHODS

Subjects

Adult subjects age 18 to 70 years sensitized to timothy grass pollen were recruited in Denver, Colo. Subjects had a history of rhinitis symptoms increasing

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Abbreviations used
Post – pre: Post 2008 value – baseline 2007 value
SLIT: Sublingual immunotherapy
tSPT: Titrated skin prick test

in June during the previous 2 years. All subjects were required to have a positive skin prick test to timothy extract 100,000 Bioequivalent allergy units (BAU)/mL containing 680 μg Phl p 5 (ALK-Abelló, Round Rock, Tex) with a wheal of ≥5 mm in diameter. Skin prick tests to a total of 12 allergen extracts were performed by using the prick method with a DuoTip (Lincoln Diagnostics, Decatur, Ill), and antihistamines were withheld 7 days before skin testing and nasal challenge testing. Subjects with mild intermittent asthma were enrolled if they were well controlled as defined by National Heart, Lung, and Blood Institute criteria and on short-acting β-agonists only.[13] Subjects with asthma had spirometry performed at the screening visit and were required to have an FEV_1 ≥80% predicted. Subjects who had received immunotherapy to grass pollen extract within the last 5 years were excluded. Exclusion criteria also included subjects using nasal corticosteroids, systemic corticosteroids, or decongestant sprays within 1 month before baseline. Antihistamines, leukotriene receptor antagonists, and tricyclic antidepressants were also withheld during the baseline period and grass season, except for the allowed study medications. Subjects were excluded if they were pregnant, not using an appropriate method of birth control, or taking β-blockers or monoamine oxidase inhibitors. Subjects older than 50 years had additional safety screening procedures (see this article's Online Repository at www.jacionline.org). The study was conducted under an Investigational New Drug Application issued by the US Food and Drug Administration, and the Institutional Review Board of National Jewish Health approved the study. All subjects signed an approved informed consent before participating.

Study design

This was a single-center, randomized, double-blind, placebo-controlled study. Participants were enrolled for 15 months. Patients were screened and followed through the grass pollen season of 2007, recording their symptoms and rescue medication use. Subjects were assessed in July after the grass pollen season, and subjects who met inclusion criteria were treated for 10 months with SLIT (September 2007 to July 2008). Subjects were instructed to take SLIT each morning on an empty stomach and to hold the solution under the tongue for 2 minutes and not to eat or drink for 15 minutes after the dose. Subjects initially used dropper bottles (Apothecary Products, Burnsville, Minn) to measure 0.25 mL SLIT, and later, when they became available, switched to pump glass bottles (Greer Laboratories, Lenoir, NC) that dispensed 0.28 mL SLIT every morning. The 10-mL vial from which the treatment dose was administered contained for timothy monotherapy SLIT 1 mL of 100,000 BAU/mL timothy extract containing approximately 680 μg/mL Phl p 5 and 9 mL 50% glycerinated saline diluent (ALK-Abelló), making up a daily dose of approximately 19 μg Phl p 5. The treatment extract for the multiple allergen group contained the same amount of timothy plus 1 mL each of an additional 9 unstandardized extracts 1:20 wt/vol in 50% glycerin: maple, ash, juniper, American elm, cottonwood, *Kochia*, ragweed, sagebrush, and Russian thistle (ALK-Abelló). The same extracts were included for each subject in the multiple allergen mix without regard to whether they were sensitive to the extract on skin prick testing. The cumulative monthly dose in the 2 active groups for timothy was 571 μg Phl p 5 (approximately 30 times the standard customary SCIT dose), and in the multiallergen group, the cumulative monthly dose for the nonstandardized allergens was approximately 15 to 20 times the standard customary SCIT dose. The placebo group received caramelized sugar with glycerinated saline diluent to mimic the color of the active treatment groups. Caramelized sugar was also added to the timothy monotherapy extract to mimic the color of the multiple allergen mix. Histamine was not added to the placebo or timothy monotherapy extracts because preliminary studies revealed no symptoms with sublingual administration of histamine 1 mg/mL.

Clinical assessments

All subjects completed diary cards recording symptom and medication scores from May 14 to July 1 of the grass season of both the observational year and the treatment year. Subjects also underwent titrated nasal challenges with timothy extract, titrated skin prick testing to timothy extract, and phlebotomy for laboratory analysis of timothy-specific IgE, timothy-specific IgG_4, and secreted cytokines from stimulated PBMCs at baseline (July 2007) and after treatment (July 2008). During the grass season, subjects were provided with pseudoephedrine 30 mg for their rhinitis symptoms to be taken as often as 4 times daily. If their symptoms were not controlled with pseudoephedrine, they were allowed to take loratadine 10 mg once daily, and if their symptoms were severe and not controlled with both these medications, they were allowed to use an intranasal steroid of their choice (maximum dose twice daily).

Subjects self-administered SLIT daily at home. They returned to the clinical research center on a monthly basis initially and then every other month to review side effects and compliance, and to receive a new supply of extract. The first dose of each new bottle was given in the Weinberg Clinical Research Unit under the supervision of a physician, and the subjects were monitored for 30 minutes after their dose. Because this was the first SLIT trial with multiple allergen extracts administered at high doses, a cautious build-up was employed. The first month of therapy included a 4-week build-up phase in which patients received 1:1000 of the maintenance dose the first week, 1:100 of the maintenance dose the second week, 1:10 of the maintenance dose the third week, and maintenance dose for the next week and the remainder of the study. During build-up, subjects were to resume their previous tolerated dose if side effects of urticaria, angioedema, or wheezing developed. At each visit, subjects were queried regarding symptoms and adherence, received new SLIT treatment bottles, and returned their old bottles, which were weighed for compliance.

Symptom and medication scoring

Details are provided in the Online Repository.

Titrated nasal challenge

Titrated nasal challenge with timothy extract was performed at baseline in July 2007 and after 10 months of SLIT treatment in July 2008 by using a modification of the method of Bousquet et al.[14] The nasal challenges were performed by spraying 0.1 mL solution into each nostril at 10-minute intervals (nasal spray bottles from PharmaSource, Centennial, Colo). The first dose contained saline and subsequent doses contained half-log[10] increasing concentrations of timothy extract beginning from 3.3 BAU/mL and increasing to 100,000 BAU/mL. Symptoms were scored by the scoring system of Bousquet et al.[14] Briefly, symptoms such as sneezing, rhinorrhea, nasal congestion, pruritus, and conjunctivitis were assigned a number between 0 and 3. Patients were asked to rate their symptoms after each incremental dose until a noncumulative score of 5 was achieved, which was the threshold dose.

Titrated skin prick tests

Titrated skin prick tests (tSPTs) were performed in duplicate on the subject's back. The same dilutions of timothy extract used in the nasal challenges were used for the titrated SPTs. These were prepared by performing serial 10-fold dilutions of both 100,000 BAU/mL and 33,000 BAU/mL timothy extract. Concentrations were increased until at least 1 dilution produced a wheal <5 mm and 1 dilution produced a wheal >5 mm. A line was fit through these dilutions, and the concentration producing a 5-mm wheal was calculated. tSPTs were performed by S.M.A., who achieved a coefficient of variation of 16.25 on repetitive testing with histamine on a single patient. The mean and SD of the histamine controls were 3.49 mm and 1.39 mm, respectively.

Pollen counts

Pollen counts were performed 5 days per week by using a Burkhard Spore Trap (Rickmansworth, United Kingdom). Counts were converted to and expressed as pollen grains per cubic meter.

Timothy-specific immunoglobulin measurements

Serum was obtained before and after completion of SLIT in July 2007 and July 2008. Undiluted samples were analyzed for allergen-specific IgE by means of Phadia ImmunoCAP system (IBT Laboratories, Lenexa, Kan). Serum samples diluted 1:1000 were used to measure timothy-specific IgG_4 using the Phadia ImmunoCAP system-specific IgG_4 FEIA (IBT Laboratories).

Proliferation assay (cytokine induction)

Details are provided in the Online Repository.

Cytokine measurement

Details are provided in the Online Repository.

Statistical analysis

Baseline and posttreatment variables are summarized in means (SDs) or counts (percentages). Outcome variables were analyzed as absolute change from baseline (post – pre). Categorical variables were compared by using χ^2 tests, whereas outcome variables and continuous baseline variables were compared between groups by using 1-way ANOVA; pairwise group comparisons were made by using *t* tests. The false-positive type 1 error rate was controlled for multiple comparisons by using the Hochberg adjustment, where the largest pairwise *P* value is compared to .05 and the smallest is compared to .05/3 = .02. For all variables except symptom scores, pre and post scores were logarithmically transformed to approximate model assumptions better. Statistical significance was defined as a *P* value of .05 or less, and all reported *P* values are based on 2-sided tests.

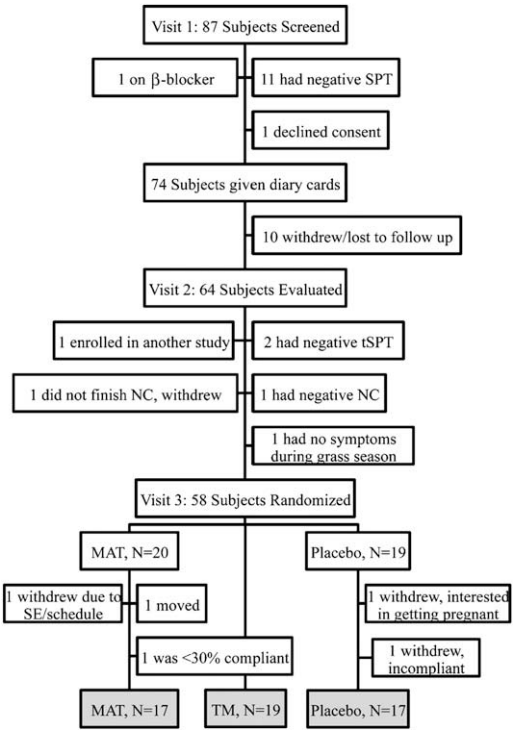

FIG 1. Flow chart of subjects. *MAT,* Multiallergen treatment; *N,* number of subjects; *NC,* nasal challenge; *SE,* side effects; *SPT,* skin prick test; *TM,* timothy monotherapy.

RESULTS

Eighty-seven subjects were screened (Fig 1, or see the Online Repository for details). Fifty-eight patients met inclusion criteria and were randomly assigned to 1 of 3 treatment groups: placebo, SLIT with timothy extract alone as monotherapy, or SLIT with the same dose of timothy extract plus 9 additional allergens (Fig 1, or see the Online Repository for details). Of the 54 subjects who completed the study, 1 subject in the multiallergen group had less than 40% compliance with her SLIT therapy, so her results were not included in the analysis. All other subjects reported greater than 80% compliance.

There were no significant differences detected across treatment groups in the baseline demographics of the 53 subjects included in the final analysis. The mean age, baseline symptom scores, medication scores, nasal challenge results, tSPT results, timothy-specific IgE levels, and timothy-specific IgG_4 levels were comparable in all 3 arms (Table I).

The normal precipitation for the first 6 months of the year in Denver is 8.09 inches. The precipitation for this period in 2007 was 6.44 inches and in 2008 only 3.04 inches. Because of this record low rainfall, the grass pollen counts in Denver 2008 were much lower than in the observational season of 2007 (Fig 2, and see the Online Repository), and thus, all 3 groups had improved medication and symptom scores during the second grass pollen season.

All subjects achieved the projected maintenance dose and hence received the same maintenance and total cumulative dose of timothy extract. For the primary outcomes, there was no significant difference in the post−pre symptom scores ($P = .96$) or medication scores ($P = .7$) among the 3 groups (Fig 3; see Table E3 in the Online Repository for rescue medication use). In contrast, there were significant differences in the results of the nasal challenge and tSPTs (Fig 4 and 5). Subjects who were treated with timothy monotherapy had significantly improved nasal challenge results compared with placebo ($P = .03$), but there was no significant difference in nasal challenge results in the multiallergen group compared with placebo ($P = .11$). Both the timothy monotherapy group ($P = .001$) and the multiallergen group ($P = .04$) had significantly improved tSPT results compared with placebo, although the improvement in the monotherapy group was quantitatively greater. In addition, timothy-specific IgE significantly increased in both the timothy monotherapy group ($P = .008$) and the multiallergen group ($P = .02$) compared with placebo. Timothy-specific IgG_4 levels increased only in the timothy monotherapy group compared with placebo ($P = .005$; Fig 6). There was also a significant decrease in IFN-γ levels measured after lymphocyte proliferation with timothy extract containing 20 μg Phl p 5 in the timothy monotherapy group (–0.75; $P = .02$) compared with placebo but not in the multiallergen group (–0.62; $P = .09$) compared with placebo (–0.27). There were no significant differences detected in the levels of the other measured secreted cytokines including IL-4, IL-5, and IL-10 among the 3 groups (see Table E4 in the Online Repository). In addition, there were no significant differences between the timothy monotherapy and multiallergen therapy groups in any of the primary or secondary outcomes measured. All significant comparisons remained significant after adjustment for multiple comparisons.

There were significantly more adverse events in both active groups compared with placebo (see Table E2 in the Online Repository). In the timothy monotherapy group, 84% of subjects experienced adverse events ($P < .0001$), and in the multiallergen group, 65% of subjects experienced adverse events ($P = .0003$) compared with placebo (6%). Once again, there were no significant differences in the number of adverse events in the timothy monotherapy and multiallergen groups ($P = .18$). Most of the adverse events were mild in nature and included itching, burning,

TABLE I. Demographic data and primary and secondary outcomes of the 53 subjects

	TM	MAT	Placebo	*P* value
Subjects (N)	19	17	17	
Age (y), mean (95% CI)	39.3 (34, 44)	35.6 (30, 41)	38.8 (33, 44)	.57
Sex, N (%)				
Male	5 (26.3)	7 (41.2)	8 (47.1)	.40
Female	14 (73.7)	10 (58.8)	9 (52.9)	
Asthma, N (%)	3 (15.8)	2 (11.8)	0 (0)	—‡
Average SS, mean (95% CI)				
Baseline §	6.3 (4.3, 8.2)	8.1 (6.0, 10.2)	6.4 (4.3, 8.4)	.36
Post	4.0 (2.0, 5.9)	5.4 (3.3, 7.5)	3.9 (1.9, 5.9)	.50
Post – pre	–2.3 (–4.1, –.40)	–2.7 (–4.7, –.67)	–2.4 (–4.4, –.50)	.96
Average MS, mean $\log_{10}$ (95% CI)				
Baseline	.19 (.09, .29)	.17 (.06, .27)	.11 (.01, .21)	.48
Post	.10 (.02, .17)	.07 (–.01, .15)	.05 (–.03, .13)	.69
Post – pre	–.09 (–.17, –.02)	–.10 (–.18, –.02)	–.06 (–.13, .02)	.70
NC threshold concentration, mean $\log_{10}$ BAU/mL (95% CI)				
Baseline	1.9 (1.5, 2.3)	1.7 (1.3, 2.2)	2.3 (1.9, 2.7)	.14
Post	2.5 (2.0, 3.1)	2.2 (1.6, 2.8)	2.1 (1.6, 2.7)	.55
Post – pre	.65 (.12, 1.2)* $P = .02$	.46 (–.10, 1.0)	–.17 (–.72, .37)	.09
Geometric mean of difference BAU/mL	237	108	–73	
tSPT, threshold concentration $\log_{10}$ BAU/mL (95% CI)				
Baseline	2.3 (1.8, 2.7)	2.5 (2.1, 3.0)	2.7 (2.2, 3.2)	.36
Post	3.2 (2.8, 3.6)	3.1 (2.7, 3.6)	2.8 (2.3, 3.2)	.33
Post – pre	.95 (.60, 1.3)* $P = .001$	.64 (.26, 1.0)† $P = .03$	.05 (–.34, .44)	.005
Geometric mean of difference BAU/mL	1385	943	130	
Timothy-specific IgE, mean $\log_{10}$ KU/L (95% CI)				
Baseline	.93 (.67, 1.2)	.93 (.65, 1.2)	.81 (.53, 1.1)	.78
Post	1.0 (.73, 1.3)	1.0 (.73, 1.3)	.75 (.45, 1.0)	.34
Post – pre	.07 (–.005, .15)* $P = .02$	.10 (.01, .18)† $P = .008$	–.06 (–.14, .02)	.02
Geometric mean of difference KU/L	1.49	1.49	–.83	
Timothy-specific IgG_4, mean $\log_{10}$ μg/mL (95% CI)				
Baseline	.09 (.02, .16)	.08 (.006, .15)	.12 (.05, .19)	.68
Post	.14 (.06, .22)	.11 (.02, .19)	.11 (.03, .19)	.8
Post – pre	.05 (.02, .07)* $P = .005$	.03 (–.002, .05)	–.01 (–.04, .02)	.02
Geometric mean of difference μg/mL	.23	.09	–.03	

MAT, Multiallergen therapy; *MS,* medication score; *NC,* nasal challenge; *SS,* symptom score; *TM,* timothy monotherapy.
*Statistical significance in pairwise comparison using the *t* test between TM and placebo.
†Statistical significance in pairwise comparisons using the *t* test between MAT and placebo.
‡No comparison made because of 0 cell.
§Baseline was June 1 to 28, 2007, for SS and MS and July 2007 for the remainder. Post was the same dates in 2008. Post – pre = post 2008 value – baseline 2007 value.

irritation, numbness, and tingling sublingually or in the mouth. Other side effects included swelling of the sublingual area or mouth, sore throat, cold sores, hay fever symptoms, heartburn, and nausea. None of the subjects experienced urticaria, bronchoconstriction, or other systemic symptoms during the study. As noted, 1 subject dropped out of the multiallergen group because of adverse effects of persistent lip and mouth swelling.

DISCUSSION

In this study, which compares timothy grass SLIT as monotherapy with timothy grass SLIT combined with 9 other allergen extract and with placebo, there were significant improvements in the nasal challenge scores, tSPT results, and timothy-specific IgG_4 levels in the timothy monotherapy group compared with

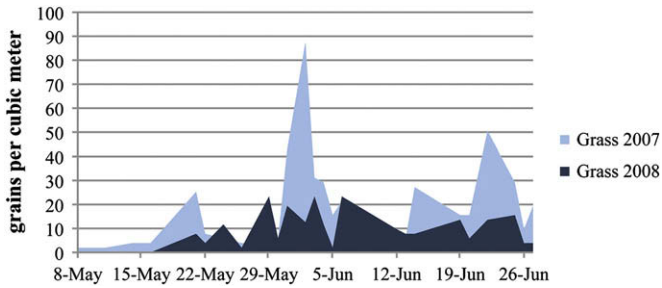

FIG 2. Grass pollen counts, 2007, 2008.

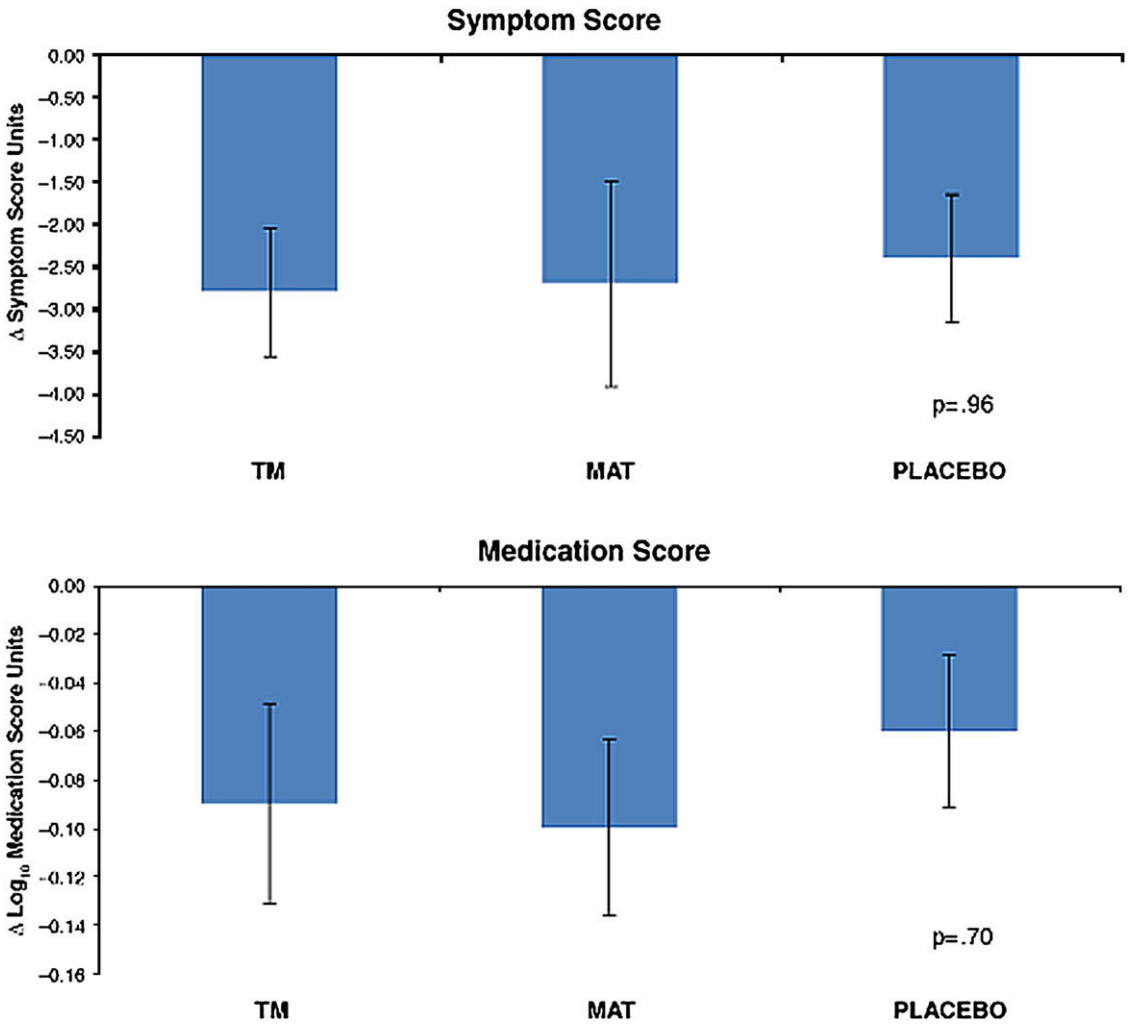

FIG 3. Mean (2008 minus 2007) reduction in average symptom scores and mean reduction in $\log_{10}$ average medication scores. Graphed values are in the upper figure the mean difference between posttreatment and baseline-years for the average symptom scores ± 1 SE, and in the lower figure the mean difference between $\log_{10}$post minus $\log_{10}$baseline-year average medication scores ± 1 SE. Maximum possible symptom score was 24. Absolute values are given in Table I. *P* values are derived from ANOVA of the 3 scores. *MAT,* Multiallergen therapy; *TM,* timothy monotherapy.

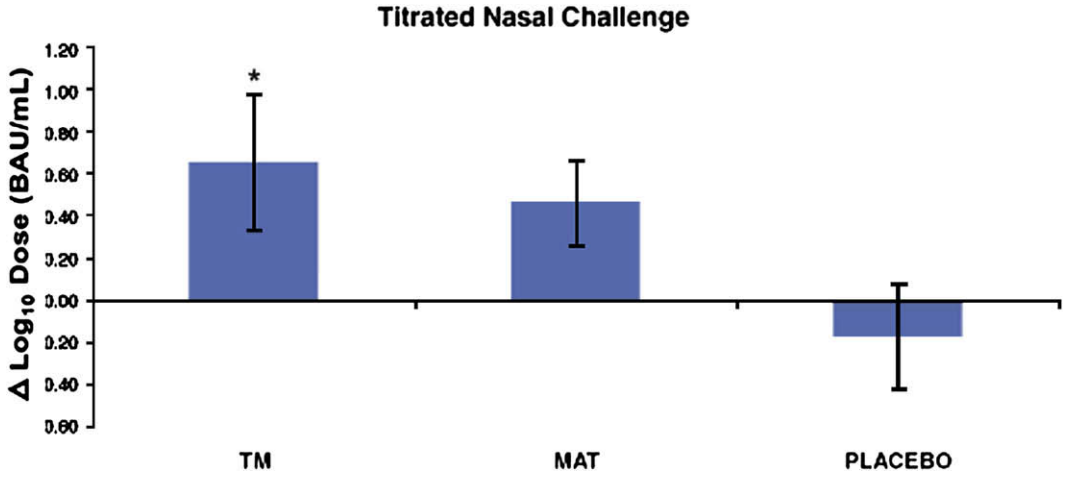

FIG 4. Mean change (2008 minus 2007) in $\log_{10}$ nasal challenge scores. Graphed values are the mean difference between $\log_{10}$post minus $\log_{10}$baseline-year threshold doses ± 1 SE, TM vs placebo. *MAT,* multiallergen therapy; *TM,* timothy monotherapy. *$P = .03$, TM vs placebo.

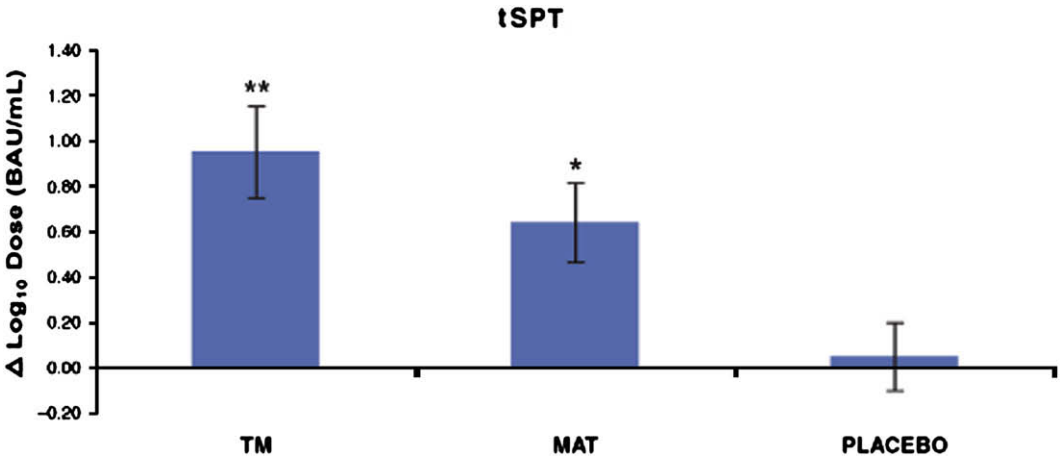

FIG 5. Mean change (2008 minus 2007) in $\log_{10}$ tSPT results. Graphed values are the mean difference between $\log_{10}$post minus $\log_{10}$baseline-year mean threshold doses for tSPT ± 1 SE. *MAT,* Multiallergen therapy; *TM,* timothy monotherapy. ***P* = .001, TM vs placebo; **P* = .04, MAT vs placebo.

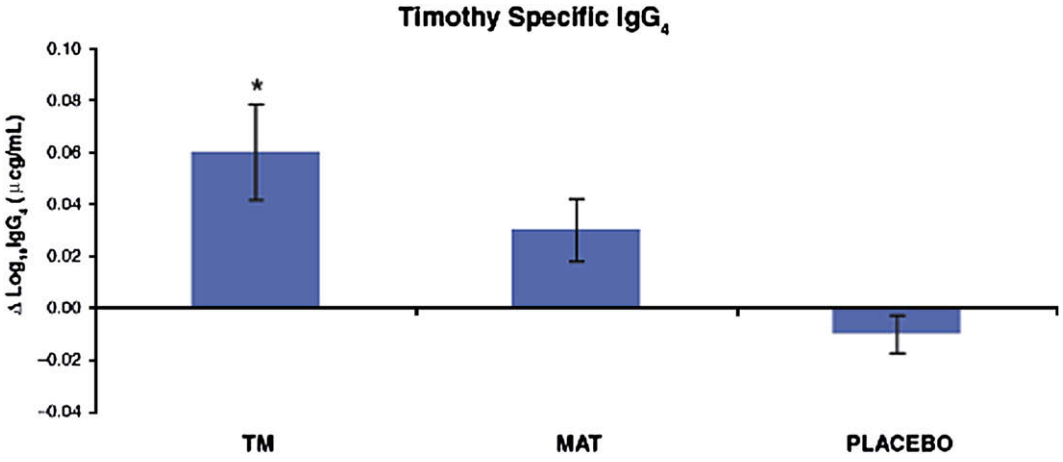

FIG 6. Mean change (2008 minus 2007) in $\log_{10}$IgG$_4$ results. Graphed are the mean differences between $\log_{10}$post minus $\log_{10}$baseline-year timothy-specific IgG$_4$ titers ± 1 SE. *MAT,* Multiallergen therapy; *TM,* timothy monotherapy. **P* = .005, TM vs placebo.

placebo. In addition, there were significantly reduced IFN-γ levels from allergen-stimulated PBMCs in the timothy monotherapy group compared with placebo. In the multiallergen group, there was also a significant but quantitatively smaller improvement in tSPT results compared with placebo, but no differences in the other outcomes measured. Both active treatment groups experienced more adverse events compared with placebo.

The treatment year of 2008 was close to the driest year ever recorded in Denver, with significantly decreased grass pollen counts. The low grass pollen counts in the treatment year may have contributed to the lack of significant differences in the symptom and medication scores among the 3 groups. For instance, there were days in the observational year 2007 when the grass pollen counts ranged from 42 to 87 grains per cubic meter, which is typical of Denver summers. In contrast, the highest grass pollen counts in 2008 reached 23 grains per cubic meter, with most days having less than 15 grains per cubic meter.

There were, however, changes in the timothy monotherapy group that have been shown in other studies to be indicative of a favorable clinical response to immunotherapy. tSPT, an objective measure not dependent on pollen counts, showed significant improvement after active SLIT treatment. tSPT endpoints have been shown to be a significant outcome measure in accessing the efficacy of immunotherapy treatment, with thresholds after immunotherapy correlating with symptoms on subsequent natural pollen exposure.[15] Although there was not a significant difference between the 2 active groups in the outcomes of the tSPTs, the magnitude of the improvement was greater in the timothy monotherapy group than the placebo group.

Another outcome not dependent on pollen counts was the titrated nasal challenge scores. Titrated nasal challenges significantly improved in the timothy monotherapy group compared with placebo. There was no difference in nasal challenge results between the multiallergen and placebo groups. Titrated nasal challenges after immunotherapy have been shown to be an important measure of immunotherapy efficacy and to correlate with symptom and medication scores during natural pollen exposure.[15]

The lymphocyte stimulation studies showed a significance decrease in IFN-γ levels in the timothy monotherapy group

compared with placebo. The decreased IFN- γ levels observed in the active monotherapy group may be a result of upregulation of regulatory T-cell activity in early SLIT, which has been recently reported.[16] Conversely, there were no significant differences in the other cytokines measured including IL-4, IL-5, and IL-10. This may be a result of the small sample size or the use of less sensitive assays with the Luminex system (Luminex Corp, Austin, Tex) compared with other laboratory methods. No differences from placebo were observed in secreted cytokines in the multiple allergen group.

There were also observed differences in timothy-specific antibody levels in the active SLIT groups. The increased timothy-specific IgG_4 levels observed in the timothy monotherapy group may also reflect regulatory T-cell activity. Specific IgG_4 levels have been shown to be correlated with successful immunotherapy.[17] There was no significant improvement in IgG_4 levels in the multiallergen group compared with placebo, suggesting greater efficacy in the timothy monotherapy group compared with the multiallergen treatment. The increased timothy-specific IgE levels seen in both the timothy monotherapy group and multiallergen group are what is typically observed in early immunotherapy.

The purpose of our study was to determine whether SLIT is effective in the treatment of patients with grass allergy by using US Food and Drug Administration–standardized timothy extract, and whether this benefit is reduced by combination with other allergens. In our results, we found significantly reduced sensitivity on titrated skin prick testing, increased threshold on nasal challenge, increased timothy-specific IgG_4 levels, and decreased secretion of IFN-γ from allergen stimulated PBMCs in the timothy monotherapy group compared with placebo. On the other hand, the only improvement observed with multiallergen treatment compared with placebo was a reduced sensitivity on titrated skin prick testing, and the change was quantitatively less than that seen in the timothy monotherapy group. There were no significant differences between the timothy monotherapy and the multiallergen groups in any primary outcome measures. The lack of clear differences between the 2 active groups may be a result of the small number of subjects in the study. We performed a power calculation based on 2007 baseline and 2008 posttreatment symptoms. On the basis of these data, demonstration of a 15% of baseline difference in the response to timothy monotherapy compared with timothy multiallergen therapy would have required 113 subjects in each treatment arm in 2007 and 213 subjects in each treatment arm in 2008. However, in every outcome measure, the magnitude of response to the timothy monotherapy group was greater than in the multiallergen group. Also, significant changes in nasal challenges and timothy-specific IgG_4 levels, both markers of successful immunotherapy, were not observed in the multiallergen treatment group. These observations clearly suggest interference with the effectiveness of timothy SLIT when multiple allergens are coadministered. Limitation in the sublingual absorptive capacity has been suggested in a previous publication in which daily low-dose SLIT was superior to a higher SLIT dose regimen 3 times a week.[12] Alternative hypotheses include a limited number of macrophages in the sublingual tissue. Antigen competition is less likely, because the subjects were often not sensitive to the additional pollen extracts administered. Larger studies with multiallergen treatment will be required to explore this possible mechanism further.

In conclusion, the results of this study showed that clinically relevant responses can be achieved with a US-standardized timothy extract administered in a high dose over a period of 10 months. A similar dose of timothy extract combined with 9 other pollen extracts showed a very limited response. These results raise questions regarding the use of multiple allergen mixes for SLIT.

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Clinical implications: The clinical efficacy of SLIT may be reduced with the addition of multiple allergens, potentially limiting its use in polysensitized individuals.

REFERENCES

1. Schatz M. A survey of the burden of allergic rhinitis in the USA. Allergy 2007; 62(Suppl 85):9-16.
2. Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. Cochrane Database Syst Rev 2007 (1):CD001936.
3. Wilson DR, Torres LI, Durham SR. Sublingual immunotherapy for allergic rhinitis. Cochrane Database Syst Rev 2003;(2):CD002893.
4. Marogna M, Spadolini I, Massolo A, Zanon P, Berra D, Chiodini E, et al. Effects of sublingual immunotherapy for multiple or single allergens in polysensitized patients. Ann Allergy Asthma Immunol 2007;98:274-80.
5. Esch RE. Specific immunotherapy in the U.S.A.: general concept and recent initiatives. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M 2003;94: 17-22.
6. Lowell FC, Franklin W. A double-blind study of the effectiveness and specificity of injecton therapy in ragweed hay fever. N Engl J Med 1965;273:675-9.
7. Lowell FC, Franklin W. Comparison of two dosages of ragweed extract in the treatment of pollenosis. JAMA 1967;201:915-7.
8. Johnstone DE, Crump L. Value of hyposensitization therapy for perennial bronchial asthma in children. Pediatrics 1961;27:39-44.
9. Adkinson NF Jr, Eggleston PA, Eney D, Goldstein ED, Schuberth KC, Bacon JR, et al. A controlled trial of immunotherapy for asthma in allergic children. N Engl J Med 1997;336:324-31.
10. Dunsky EH, Goldstein MF, Dvorin DJ, Belecanech GA. Anaphylaxis to sublingual immunotherapy. Allergy 2006;61:1235.
11. Eifan AO, Keles S, Bahceciler NN, Barlan IB. Anaphylaxis to multiple pollen allergen sublingual immunotherapy. Allergy 2007;62:567-8.
12. Bordignon V, Parmiani S. Variation of the skin end-point in patients treated with sublingual specific immunotherapy. J Investig Allergol Clin Immunol 2003;13:170-6.
13. Busse WW. National Asthma Education and Prevention Program Expert Panel Report 3: guidelines for the diagnosis and management of asthma: summary report 2007. J Allergy Clin Immunol 2007;120:S94-138.
14. Bousquet J, Lebel B, Dhivert H, Bataille Y, Martinot B, Michel FB. Nasal challenge with pollen grains, skin prick tests and specific IgE in patients with grass pollen allergy. Clin Allergy 1987;17:529-36.
15. Bousquet J, Maasch H, Martinot B, Hejjaoui A, Wahl R, Michel FB. Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids, II: comparison between parameters assessing the efficacy of immunotherapy. J Allergy Clin Immunol 1988;82(3 Pt 1):439-46.
16. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. J Allergy Clin Immunol 2007; 120:707-13.
17. Gehlhar K, Schlaak M, Becker W, Bufe A. Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome. Clin Exp Allergy 1999;29:497-506.

ADDITIONAL SAFETY ASSESSMENTS FOR SUBJECTS OLDER THAN 50 YEARS

For subjects older than 50 years, additional safety assessments were conducted, including serum fasting glucose, serum fasting cholesterol, and an EKG. If these were abnormal, the subjects were excluded from the study. In addition, patients older than 50 years with any history of ischemic disease, aortic aneurysm, hypertension, diabetes (unless they had a normal stress test/angiography in the past year), smoking, hyperlipidemia, or family history of coronary artery disease were excluded.

SYMPTOM/MEDICATION SCORING

Subjects recorded symptom scores and rescue medication use twice daily during the grass pollen season at baseline and while on SLIT treatment. Subjects rated symptoms of runny nose, stuffy nose, itchy nose, sneezing, itchy eyes, teary eyes, red eyes, and itchy ears/throat on a scale of 0 to 3. A score of 0 represented no symptoms, 1 was mild symptoms, 2 was moderate symptoms, and 3 was severe symptoms. In the evening, subjects were instructed to record their symptoms reflecting the worst part of their day. In addition, subjects recorded pseudoephedrine, loratadine, and/or nasal steroid use on a daily basis. Daily symptom and medication scores were averaged each week. Medication use was calculated with the following prespecified scores: pseudoephedrine, 1 point; loratadine, 4 points; and nasal steroids, 8 points per dose. The relative values were based on placebo-controlled trials of the individual classes of drugs for treatment of seasonal allergic rhinitis. To calculate average symptom and medication scores for each season, the total symptom or medication scores for each of the 4 weeks were calculated, added together, and divided by 4.

PROLIFERATION ASSAY

PBMCs were isolated using a Ficoll-Hypaque density gradient centrifugation from heparinized venous blood. Cells were washed and resuspended at 1×10^6 cells/mL in RPMI (Cellgro, Herndon, Va) with 5% type AB human sera, penicillin-streptomycin, and L-glutamine. Triplicate wells containing 1×10^6 PBMCs were incubated with media only or media with timothy extract containing 0.2, 2, or 20 μg/mL Phl p 5 in a 37°C, 5% CO_2 incubator for 5 days. Supernatants were collected and frozen at –70°C until analyzed.

CYTOKINE MEASUREMENT

Frozen supernatants were thawed and analyzed in duplicate by using the human cytokine LINCO*plex* premixed kit per the manufacturer's instructions (LINCO Research, Inc, St Charles, Mo). IL-4, IL-5, IL-10, and IFN-γ were measured. Briefly, antibodies specific to each cytokine are covalently coupled to microspheres, which are uniquely labeled with a fluorescent dye (Luminex Corp, Austin, Tex). The microspheres were incubated with 25-μL standards, controls, and samples overnight at 4°C; washed; then incubated with detection antibody, followed by streptavidin-phycoerythrin. After a final wash step, the microspheres were resuspended in buffer and read using the Luminex^{200} instrument. Results were analyzed by using Bio-Rad Bio-Plex software (BioRad Laboratories, Hercules, Calif) to determine the concentration of each cytokine in the samples and represent the average of duplicate samples.

POLLEN COUNTS

During June of both 2007 and 2008, there were no pollen counts $\geq$10 grains/m^3 except for grass and pine. The latter is rarely sensitizing and even less often the cause of clinical symptoms. During May, the only tree pollens recorded $\geq$10 grains/ m^3 were cedar and *Populus*. The last days with counts this high in 2007 were May 21 for both, and in 2008, May 16 for cedar and May 9 for *Populus*.

RESULTS (RECRUITMENT AND RETENTION)

Eighty-seven subjects were screened. Twenty-three subjects failed to meet initial inclusion criteria or withdrew from the study. Of these 23 subjects, 11 subjects had screening skin prick test to timothy grass extract less than 5 mm in diameter, 1 subject was on a β-blocker, 1 subject refused consent, and 10 patients withdrew consent after the screening visit. During the second visit, an additional 6 subjects failed to meet inclusion criteria and withdrew from the study. Two subjects had negative tSPT to timothy, 1 subject had a negative nasal challenge to timothy, and 1 subject did not finish the nasal challenge. Last, 1 person was involved in another study, and 1 subject had no symptoms during the baseline grass pollen season (Fig 1).

Fifty-eight patients met inclusion criteria and were randomly assigned to 1 of 3 treatment groups: placebo, SLIT with timothy extract alone as monotherapy, or SLIT with the same dose of timothy extract plus 9 additional allergens. Five subjects were monosensitized to timothy; the mean number of positive skin prick tests per subject was 5.7 (Table E1). Overall, 2 subjects dropped out of the placebo group, and 2 subjects dropped out of the multiallergen group. Fifty-four subjects completed the study. One subject dropped out of the multiallergen group because of adverse effects of persistent lip and mouth swelling, and another subject from the multiallergen group moved out of state before the final visit. In the placebo group, 1 subject dropped out because she was interested in getting pregnant, and 1 subject dropped out because of lack of follow-up and scheduling issues. Of the 54 subjects, 1 subject in the multiallergen group had less than 40% compliance with her SLIT therapy, so her results were not included in the analysis. All other subjects reported greater than 80% compliance.

TABLE E1. Skin prick test results to allergens other than timothy

Allergen extract	TM	MAT	Placebo
Maple, box elder	9	8	10
Ash, white	8	5	4
Juniper, Western	9	6	11
Elm, American	5	6	5
Cottonwood, common	6	10	8
Firebush (*Kochia*)	12	3	8
Ragweed, Western	11	10	9
Sagebrush, common	9	9	12
Russian thistle	11	10	11
Dermatophagoides farinae	6	6	2
Dermatophagoides pteronyssinus	7	7	5

MAT, Multiallergen therapy; *TM,* timothy monotherapy.

TABLE E2. Side effects reported by subjects

Side effect	Multiple allergens	Timothy monotherapy	Placebo
Oral symptoms	9	15	1
Oral swelling	1	5	0
Respiratory symptoms	3	0	0
Upset stomach/nausea	2	0	0
Heartburn	1	1	0
Cold sores	1	1	0
No symptoms	6	3	16

Recurrent symptoms were reported for 2 weeks or less by 8 subjects, for 1 month by 4, for 2 months by 7, for 3 months by 6, and for more than 3 months by 5.

TABLE E3. Subjects using rescue medication 2007/2008

Treatment group	Incomplete data	No use either year	Pseudoephedrine	Loratadine	Nasal corticosteroids	≥20 Total doses
Multiple allergens	3	2	10/4	5/3	0/0	5/1
Timothy	2	5	8/4	5/5	1/0	5/2
Placebo	0	6	8/1	3/6	1/0	3/1

First figure is the total use during 4 weeks of June 2007, and the second figure is for the same period in 2008.

TABLE E4. Cytokine release from PBMCs on stimulation with timothy extract containing 20 μg Phl p 5

Cytokines	Timothy monotherapy (pg/mL)	Multiple allergen therapy (pg/mL)	Placebo (pg/mL)	*P* value
Log_{10} IL-4 baseline	1.8 (.41)	1.6 (.51)	1.4 (.74)	.17
Post	.29 (.52)	.27 (.50)	.19 (.45)	.82
Post minus baseline	–1.5 (.56)	–1.3 (.66)	–1.3 (.79)	.49
Log_{10} IL-5 baseline	.53 (.75)	.43 (.55)	.35 (.52)	.68
Post	.09 (.21)	.05 (.21)	.08 (.21)	.88
Post minus baseline	–.43 (.61)	–.38 (.63)	–.27 (.50)	.7
Log IL-10 baseline	1.9 (.16)	2.3 (.55)	2.0 (.78)	.04
Post	.98 (.58)	1.3 (.44)	1.1 (.66)	.31
Post minus baseline	–.94 (.63)	–1.0 (.37)	–.88 (.91)	.86
Log_{10} IFN-γ baseline	.80 (.72)	.68 (.64)	.27 (.47)	.04
Post	.05 (.22)	.06 (.26)	0 (0)	Not calculated
Post minus baseline	–.75 (.67)	–.62 (.59)	–.27 (.47)	.05

All values are means and SEMs.