CLINICAL AND IMMUNOLOGICAL CORRELATES OF PRE-CO-SEASONAL SUBLINGUAL IMMUNOTHERAPY WITH BIRCH MONOMERIC ALLERGOID IN PATIENTS WITH ALLERGIC RHINOCONJUNCTIVITIS

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Sublingual immunotherapy is safe and efficacious in the treatment of patients with allergic rhinitis. The clinical and biological efficacy of modified allergens (allergoids) has not been fully clarified. We investigated in birch allergic patients the effect of a pre-co-seasonal sublingual immunotherapy regimen with a modified allergen extract on clinical parameters and on T cell proliferation and regulatory cytokine production (IL-10, TGF-beta). We found that during the birch pollen season symptoms and drug usage scores were 30 and 40% improved, respectively, in treated versus control subjects (p<0.0001 for both comparisons) whereas well days were 23.5 (33%) versus 16.9 (23%) (p=0.0024), respectively. Bet v 1 allergen specific proliferation decreased (p = 0.0010), whereas IL-10 transcription increased (p = 0.0010) in treated, but not in control patients. Moreover, TGF-beta transcription was increased, although not significantly (p=0.066), following immunotherapy. Thus, sublingual immunotherapy with modified allergen in birch-allergic subjects was safe, clinically efficacious and associated with the reduction of allergen-specific proliferation and with the increased production of the IL-10 regulatory cytokine.

Allergen-specific immunotherapy is a highly effective treatment of patients with severe allergic rhinitis, conjunctivitis and/or asthma and has been recommended by WHO as an integrated part of the allergy management strategy (1). Several controlled studies have shown that subcutaneous immunotherapy (SCIT) achieves hyposensitization and reduces both early and late responses occurring during the natural exposure to allergen. The efficacy of SCIT has been referred to the increase in allergen-specific "blocking" IgG4 antibodies (Abs) (2) and to the increased proportion of IL-10-producing T cells (3-4).

Sublingual immunotherapy (SLIT) was introduced during the 1980s as an alternative route of immunotherapy, characterized by a higher safety profile than SCIT. SLIT efficacy in reducing such clinical parameters as symptoms and medication usage has been clearly demonstrated with controlled studies in adults and children (5-7). The allergic rhinitis and its impacts on asthma (ARIA) document concluded that SLIT is a valid alternative to the injective immunotherapy and that its use in clinical practice in adults as well in children is justified (8). In a Cochrane meta-analysis, the efficacy of SLIT has been definitively proven at I-a evidence level (9).

The biological mechanisms of SLIT are still a

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matter of controversy. Indeed, it may not appear obvious how an extremely limited absorption of allergen as that obtained by the sublingual route (10-11) may significantly impact on the systemic response to allergen. However, evidence in animal models of asthma indicate that the oral route of immunotherapy is tolerogenic by redirecting the Th1/Th2 differentiation *via* induction of IL-12 synthesis by mucosal dendritic cells (12). Moreover, SLIT has been shown to impair cellular infiltration and expression of adhesion molecule expression on epithelia in target organs (13-14) and to increase systemic allergen-driven IFN-gamma (15-16) and IL-10 (17-18) production, via the up-regulation of IL18 and of the signalling lymphocytic activation molecule (SLAM) transcription factor in Th1 type cells (19).

The usage of a modified allergen in sublingual immunotherapy provides the distinct advantage of avoiding those well-known minor, but often annoying, local side effects, which can significantly affect patients' compliance to SLIT. Data on the biological modification induced by immunotherapy with modified allergens are limited (15).

Here, we evaluated clinical outcomes and immunological parameters related to regulatory T cell activity (namely, allergen-induced proliferation of T cells and production of IL-10 and TGF-beta by T cells) in birch allergic patients undergoing SLIT with a modified allergen.

Our results indicate that a short-course of SLIT with a modified allergen is clinically efficacious and can induce a decrease in allergen-specific T cell proliferation associated with an increase in cytokines capable of impairing Th2 responses. These findings encourage the usage of SLIT with modified allergens and provide the biological basis for explaining the effect of this form of treatment.

MATERIALS AND METHODS

Study design

This is an open observational, parallel-grouped, pilot study to evaluate the peripheral blood mononuclear cell (PBMC) proliferation to birch allergens and birch-induced IL-10 and TGF-beta production in PBMC from birch allergic subjects either undergoing 6 months of pre-co-seasonal sublingual immunotherapy with a monomeric allergoid of pollen from birch family (*Betulaceae*) or

receiving only drugs for symptomatic relief. A no-induction, pre-co-seasonal six-month protocol was used for immunotherapy, the clinical efficacy of which was evaluated by filing a daily diary in the symptomatic period. Patients were recruited to the study in September 2006 and started immunotherapy during November. Blood samples were taken at two outpatient visits before the start of SLIT and at the end of the immunotherapy treatment. Each treated patient received at least 8 weeks of immunotherapy before the beginning of symptoms.

Written informed consent was obtained before enrolling in the study which was performed in accordance with the Declaration of Helsinki and Good Clinical Practice. The ethics committee of the San Raffaele Institute approved the study.

Immunotherapy treatment

Eleven patients were prescribed a commercial SLIT treatment (LAIS, Lofarma Allergeni S.p.A., Milan, Italy) with a monomeric allergoid of a balanced pollen mixture of two species of the Birch family (*Betulaceae*), i.e., *Betula alba* and *Alnus incana*. Allergoid derivatives of allergens were obtained by carbamilation with potassium cyanate at alkaline pH, a reaction that leads to a substantial substitution of e-amino groups of lysine residues and consequently a strong decrease in the capacity to react with IgE antibodies (20). The product, standardized before carbamilation for allergenic potency by RAST/EAST-inhibition in comparison with an in-house reference preparation and titrated in allergenic units (AU), was formulated in orosoluble tablets and administered sublingually.

SLIT was carried out by self-administration and was continued for 6 months from October 2006 to May 2007. No build-up phase was performed, and from the first day the patients received a dose of 1000 AU (one tablets, equivalent to 3.5 μg of Bet v1), which was then taken every other day throughout the study, for six months. The cumulative dose of allergen extract received by each patient was therefore 90000 AU, totalling 315 μg of Bet v1 major allergen. The tablet had to be taken in the morning, dissolved in the mouth for 1-2 min and then swallowed.

Inclusion and exclusion criteria of treated patients and controls

Inclusion criteria were as follows: age range: 18 to 65 years and at least 2-year clinical history of significant birch pollen-induced allergic rhinoconjunctivitis (21) with or without mild asthma (22). In our experience patients with low symptom severity make it difficult to appreciate modulation of disease gravity in cohorts with low sample size; thus, all patients included in this study

had suffered symptoms so severe that anti-histamines had consistently proven to be inadequate to yield complete remission in previous years. Specific IgE against *Betula alba* were class ≥ 2 according to ImmunoCAP (Phadia, Uppsala, Sweden), skin prick test against *Betula alba* (Soluprick SQ, ALK-Abellò, Lainate, Milan, Italy) yielded a wheal diameter ≥ 3 mm and FEV_1 scored higher than 70% of predicted value. Sensitizations to *Phleum pretense*, *Parietaria judaica* pollen, olive tree, mugwort, cat dander and mites, evaluated by skin prick test (Alk-Abellò), were negative.

Exclusion criteria were as follows: significant rhinoconjunctivitis or asthma outside the birch pollen season; FEV_1 lower than 70% of predicted value at recruitment; sensitizations to allergens other than *Betulaceae* (see above); history of anaphylaxis; diagnosis of systemic autoimmune disease, with or without immunosuppressive treatment; immunotherapy with birch pollen allergen within the previous 10 years or any other allergen within the previous 5 years; symptom control with antihistamines referred as perfectly capable of cancelling any residual perception of the allergic problems; and pregnancy.

All 22 patients included in this study were clinically eligible for immunotherapy treatment on the basis of their allergic symptoms and sensitization profile, and were advised to do so. Patients were either assigned to the immunotherapy or to the control group on the basis of the self-evaluated compliance to treatment in the course of an interview aimed at exploring in detail their motivation. It is common in our clinical practice that only a portion of patients who are advised to undergo immunotherapy are sufficiently self-motivated to sustain what some of them consider a far too prolonged and demanding treatment to control a disease with a relatively short time of symptom expression. Immunotherapy treatment, in the area where these patients are living, is provided at the expenses of the public health system. Thus, in everyday clinical practice it is ethically justified to investigate the predicted compliance to treatment, before actually starting the administrative procedures to purchase the proper allergen extract at no charge to patients.

Age, sex, clinical characteristics and serological data of patients are indicated in Table I.

Assessment of symptoms and drug scores

The symptoms scores for rhinitis (rhinorrea, conjunctivitis and nasal discharge) and asthma (cough and breathlessness) were separately recorded by each patient. Each day, starting from the first day when allergic symptoms appeared, the subjects rated their rhinoconjunctivitis symptoms on a scale from 0 to 3 (0 = no symptoms, 1 = slight symptoms, 2 = moderate

symptoms, 3 = severe symptoms). The symptoms, which were separately rated, were runny nose, blocked nose, sneezing, itchy nose, gritty feeling/red/itchy eyes, and watery eyes. Subjects had free access to relief medication (cetirizine, budesonide nasal spray, ketotifene eye drops) in a stepwise fashion depending on the persistence and severity of their symptoms. Salbutamol was used in the case of asthmatic symptoms, on an "as-needed" basis. For each patient, also the total of medications taken daily was recorded in daily diary cards according to the following scale: 1 point for each application of nasal budesonide and/or ketotifene eye drops in both nostrils or eyes, respectively; 2 points for every inhalation of beta-2-agonist; 3 points for every antihistamine taken. Symptoms and medication scores were considered in each patient as the mean of the total daily scores recorded throughout the whole birch pollen season. The beginning of the pollen season was set when a concentration above 5 pollen grains per cubic meter was detected for 2 consecutive days, and lasted until it remained below this threshold for more than two days.

Well days, defined as days without intake of rescue medication and a symptom score of 2 or less, were calculated for each patient for the entire birch pollen season.

Measurement of birch-allergen specific T cell proliferation
Cell culture reagents

Complete medium used for cell cultures was prepared as follows: RPMI 1640 (PBI, Milan, Italy) was supplemented with gentamicin 5 µg/ml, glutamine 2 mM (PBI) and 5% normal human serum (Danish Red Cross Laboratories, Utrecht, Holland). As a native allergen extract, a lyophilized commercial preparations of a birch pollen mixture from Lofarma Allergeni S.p.A. was used, whose total protein concentrations was calculated using a commercial kit (Biorad laboratories, Segrate, Italy). As a purified birch major allergen, recombinant Bet v 1 (rBet v 1) was utilized (Biomay, Vienna). Endotoxin levels in the native extract and in rBet v 1, measured by the LAL test (Cape Cod, Falmouth, MA) were <0.06 EU/ml at the allergen concentrations used in the assay. As a control antigen for T lymphocytes, tetanus toxoid was used (Cannaught laboratories, Toronto, Canada).

Cells

Cell preparations containing T lymphocytes were obtained as peripheral blood mononuclear cells (PBMC) isolated by standard gradient separation (Ficoll Hypaque, Phadia, Uppsala, Sweden) from heparinated blood. Cells were cultured in complete medium with allergen (at concentrations of 20 and 5 µg/ml for birch extract and

recombinant Bet v 1, respectively) or tetanus toxoid (5 μg/ml) for 5 days in 96-well flat-bottom plates in standard 5-days proliferation assays where 200,000 cells per well were plated in 96-well flat bottom plates (Costar, PBI).

Tritiated thymidine (3H-TdR, Dupont, Cologno M., Italy) was added (1 μCi per well) in the last 6 to 18 hours of culture. Cells were harvested with a FilterMate Universal Harvester (Perkin Elmer Life Sciences, Boston, MA) and incorporated thymidine, expressed as Counts per Minute (CPM) following scintillation counting was used as a measure of allergen specific cell proliferation (TopCount NXT Microplate Scintillation and Luminescence Counter, Perkin Elmer Life Sciences). Controls were included (PBMC without antigen). The Stimulation Index (S.I.) for each antigen was calculated as the ratio between the mean 3H-TdR incorporation in test *versus* control wells. All assays were done in triplicate and mean values were used for calculation.

Measurement of IL-10 and TGF-beta transcription by T lymphocytes

PBMC cultured with antigens, as above described, were separately plated for cytokine assays in 48 wells plates (500,000 cells per well) for 48 hours. This time of culture was chosen as the most suitable to allow detection of these cytokines, on the basis of preliminary experiments where PBMC were exposed to allergen for different time periods and cytokine levels measured as below specified.

The cell pellets were collected, lysed and frozen at -80 °C in TRIzol (Invitrogen Life Technologies, Milan) until the day of RNA extraction. Before reverse transcription, RNA was DNase-treated using DNA-free (Ambion Europe, Huntingdon, UK) according to the manufacturer's instructions, 1.6 μg of oligo(dT)$_{15}$ was added, and the sample was denatured at 70°C for 10 min. RNA was reverse transcribed using 10 U of SUPER RT (HT Biotechnology Ltd, Cambridge, UK)

per microgram of total RNA, 1 SUPER RT buffer, 1 mM each deoxynucleotide triphosphate, and 40 U of RNaseOUT (Invitrogen Life Technologies) at 42°C for 40 min. Relative quantitation of specific cDNA species to beta-actin message was conducted on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using TaqMan chemistry in a multiplex PCR and the comparative threshold (*CT*) method (23) for IL-10, TGF-beta and beta-actin. Sequences for primers and probes were designed by the Assay-On-Demand Taqman® service of Applied Biosystems. Relative quantification of signal was determined by subtracting the CT for the target gene from the CT for beta-actin (DCT). Relative expression levels of cytokines for each culture condition were expressed as 2^{DCT} and cytokine stimulation indexes were calculated by elevating the log base "2" to the DCT values of the stimulated *minus* the DCT of the unstimulated culture, following the indications of the manufacturer.

Statistical analysis

The Wilcoxon's matched-pair signed-rank test and the Spearman rank order correlation test were used to evaluate whether there were differences and correlation between the compared groups of values, respectively. P values of <0.05 were considered as significant.

RESULTS

Safety and efficacy of treatment

Two patients had transient itching in their mouth, which spontaneously disappeared in less than half minute after taking the tablets. No other side effects, either systemic or local, were reported by patients who took the birch monomeric allergoid tablets, despite the unconventional protocol we used, which did not imply any induction phase, to reach

Table I. *Patients'characteristics.*

N.	Sex (M/F)	Age	IgE (pre)	IgE (post)	RC	A
Treated	11	4/7	29 (18-54)	21 (8-57)	20 (5-44)	11	4
Controls	11	6/5	31(19-59)	24 (9-57)	25 (4-61)	11	5

RC: number of patients with rhinoconjunctivitis
A: number of patients with asthma)
"IgE" indicates mean values of Betula alba specific serum IgE levels (range) as established with Phadia Immunocap (Uppsala, Sweden), which were measured before ("pre") and after ("post") the birch pollen season.
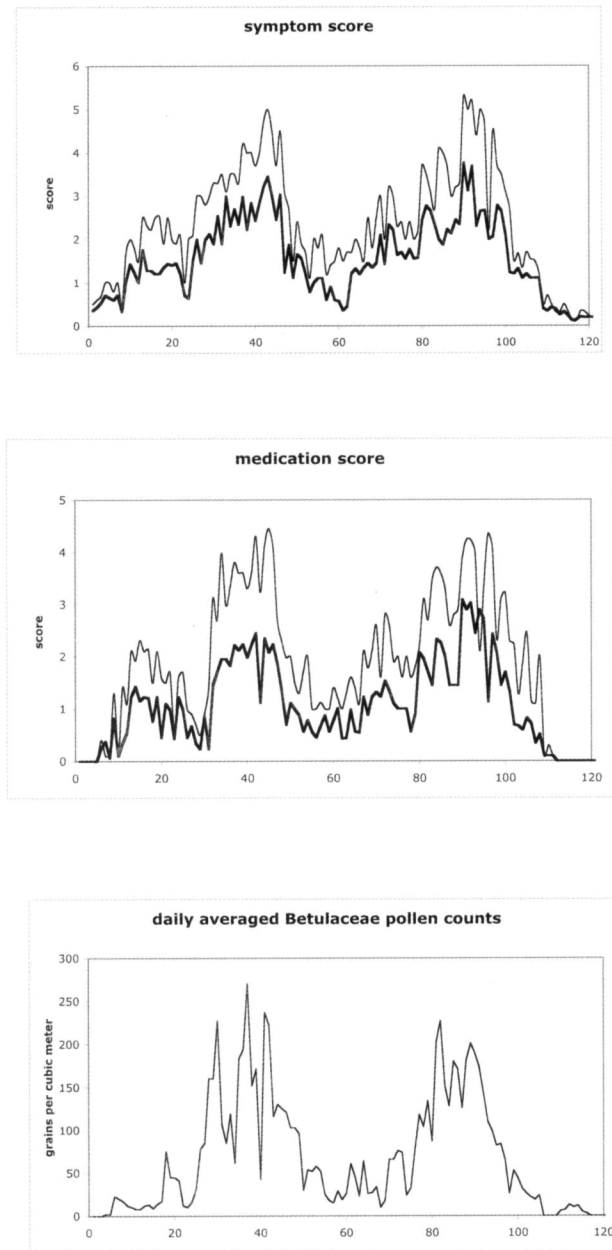
Fig. 1. *From top to bottom panel, daily averaged scoring of symptoms, scoring of rescue medication and birch pollen counts. In both the first and the second panel from top, thick and thin lines indicate treated and control individuals, respectively.*

maintenance. In particular, patients did not complain of prolonged itching in the mouth or throat, nor of gastrointestinal complaints, such as nausea and diarrhea.

Birch pollen counts were obtained from a pollen station located in the area where patients were living. As representative species of the *Betulaceae* family, pollen grains from *Betula* and *Alnus* genus were counted and their cumulative score per cubic meter was considered. The *Betullacee* pollen counts remained above 5 grains per cubic meter for 91 days (Fig. 1, bottom panel). Subjects treated with the birch monomeric allergoid scored statistically significantly lower than control subjects both in symptoms and in drug usage (Fig. 1, top and medium panel, respectively). The reduction over controls was 30% in symptom score and 40% in medication score over the entire birch pollen season ($P < 0.0001$ for both comparisons).

A well day was defined as a day when the subject did not need any rescue medication and had a symptom score no greater than 2. Subjects treated with the birch monomeric allergoid on average had 16.9 (23%) well days during the birch pollen season *versus* 23.5 (33%) in controls. Also this difference was statistically significant (p=0.0024)

Allergen specific proliferation was diminished following sublingual immunotherapy with pre-co-seasonal birch monomeric allergoid

Proliferation of PBMC stimulated *in vitro* for 5-days with tetanus toxoid, native birch extract and rBet v 1 was measured at baseline and at the end of SLIT.

A Stimulation Index (SI, i.e. the ratio between proliferation in the presence of antigen *versus* proliferation of cells in medium only) above 2 was considered as an index of specific proliferation to antigen. At baseline, a signal compatible with specific proliferation to tetanus toxoid was measured both in patients who served as controls (median value of S.I. 8.4: range 3.5-50.7) and in patients who served as treated subjects (median value of S.I. 12.8: range 3.6-27.8). This was expected, since they had been all boosted with this vaccination within the previous 5 years, and demonstrated that TT proliferation was a suitable marker for monitoring modification of the T cell response in these patients.

Proliferation to rBet v 1 and to the raw birch extract, which all patients were sensitized to, yielded a SI > 2 both in treated (21.8, range 4.9-52.3 and 22.8, range 2.4-51.1, respectively) and in control subjects (18.7, range 5.1-42.8 and 23.6, range
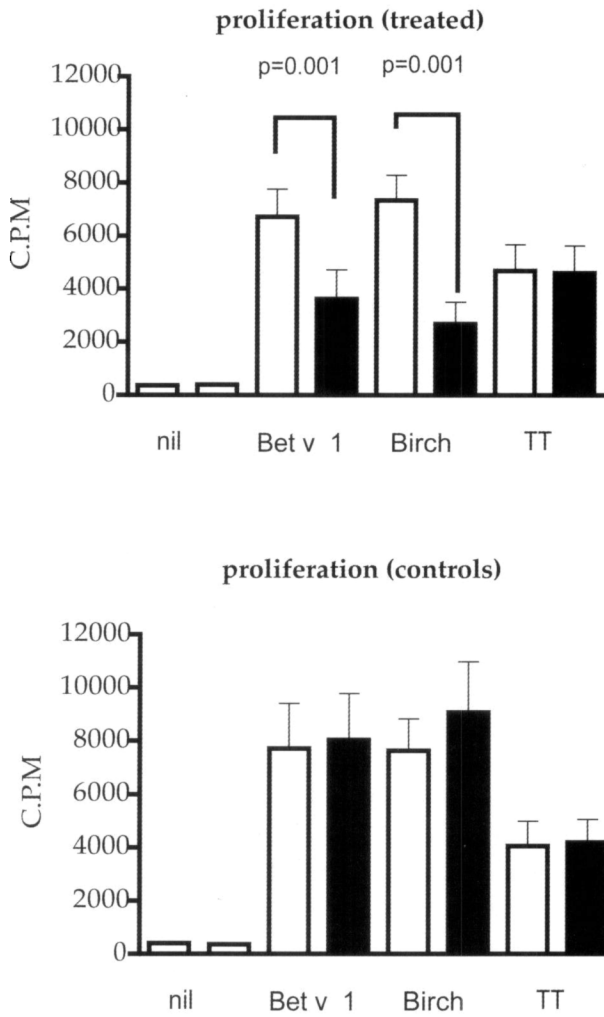
proliferation (treated)

IL-10 expression

proliferation (controls)

TGF-beta expression

Fig. 2. *Allergen-dependent T-lymphocyte proliferation in peripheral blood mononuclear cells (PBMC) from treated (top) and control subjects (bottom). Proliferation was measured with a standard 3-H-thymidine incorporation assay and expressed as count-per-minutes (CPM, on the y-axis). Columns represent mean values (± SEM) of T cell proliferation measured before the beginning of SLIT (empty columns) and after 2 months of SLIT (filled columns). Antigens used for T-cell stimulation are indicated on the x-axis. Nil = medium only. Bet v 1 = rBet v 1 major allergen. Birch = native birch extract. TT = tetanus toxoid. Results of intra-group comparisons, where significantly different, are indicated.*
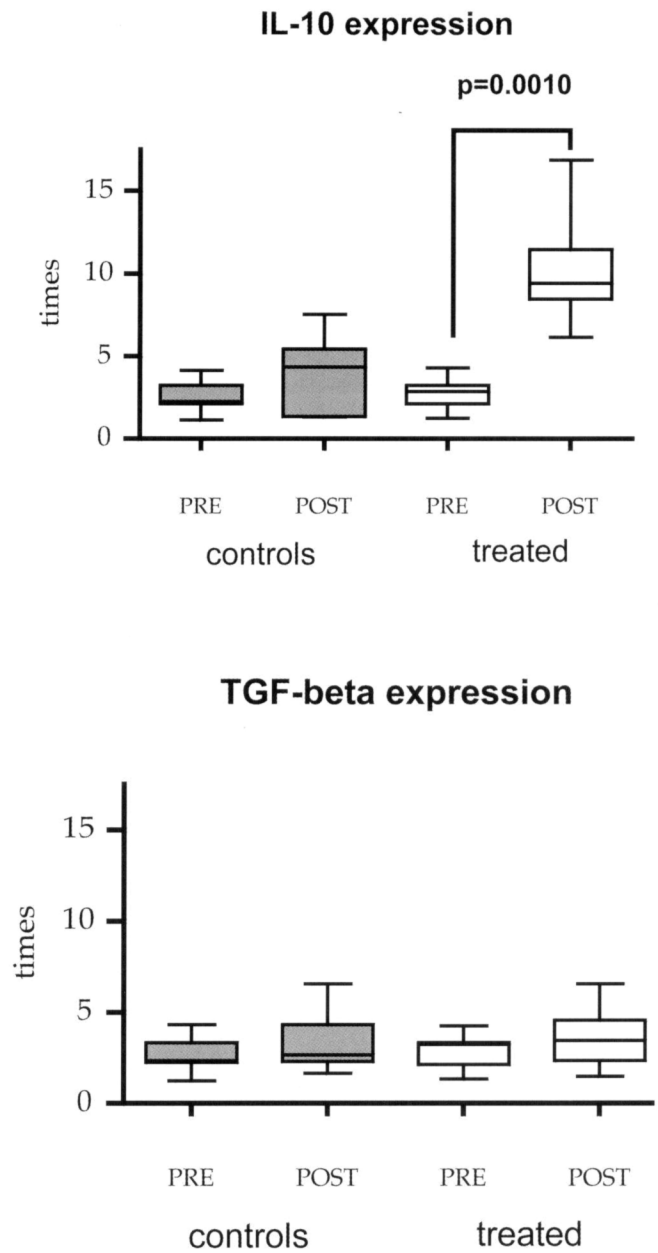
Fig. 3. *Whisker-plot representation of the median, lower and upper quartile, minimum and maximum values of IL-10 (upper panel) and TGF-beta (lower panel) expression level relative to the beta-actin control gene. Cytokine stimulation indexes (on the y-axis) were obtained by subtracting the expression levels for the target gene (relative to beta-actin) in stimulated versus unstimulated cultures. Whisker plots indicate values measured before (PRE) and after SLIT (POST). In each panel results obtained with treated and control subjects are depicted side by side. The result of intra-group comparison, where significantly different, is indicated.*

2.6-60.8, respectively). This result showed that all patients included in the study had a measurable specific T cell immune response to the allergen they were sensitized to.

After immunotherapy, mean values of proliferation to rBet v 1 and to raw birch extract were 45.4 and 62.5% diminished (p = 0.0010 for both comparisons) (Fig. 2, top panel). In contrast, no differences were observed for proliferation to rBet v 1 and to raw birch extract in untreated, control individuals (Fig. 2, bottom panel).

As a further control for the specificity of the effect of birch immunotherapy on allergen specific T cell proliferation, no differences were observed in proliferation of T cells to TT either in controls or in treated subjects (Fig. 2).

Transcription of IL-10 and TGF-beta in rBet v 1 stimulated lymphocytes is increased following sublingual immunotherapy

Transcription of IL-10 and TGF-beta were measured in 3-day rBet v 1 stimulated PBMC before and after immunotherapy, by means of quantitative PCR. In untreated (control) subjects, the mean values of the gene specific signal were 1.56 and 1.23 higher in the second (end of the season) determination as compared to baseline for IL-10 and TGF-beta, respectively (Fig. 3), but neither of these comparisons reached a significant value. In subjects who underwent IT, the mean values of the gene specific signal were 3.8 and 1.26 time increased as compared to baseline for IL-10 (p= 0.0009) and TGF-beta (p = 0.066), respectively (Fig. 3).

DISCUSSION

In the present study we report that the proliferation of allergen-stimulated T lymphocytes was decreased and the transcription of IL-10 was augmented after a pre-co-seasonal 6-months course of SLIT with a modified allergen in birch allergic individuals, but not in birch allergic patients, who only used symptomatic drugs. Moreover, TGF-beta transcription, which can also be attributed to regulatory T cells, were augmented in allergen stimulated PBMC following immunotherapy, and this increase was barely non-significant (p = 0.066). The pattern of the modification of the considered biological markers

is consistent with an increased regulatory T cell activity and is in agreement with a previous report from our group (24). The evaluation of symptom and drug consumption scores indicated that treated, but not control subjects, had a significantly improved symptom control, which paralleled exposure to the number of birch pollen grains in the area where patients were living. No SLIT-related adverse events were reported, including local oral symptoms, despite the fact that a no-induction, simplified protocol was used to facilitate patients' adherence to treatment.

The extent of clinical improvement we observed in treated patients (30.0 and 40.0%, for clinical scores and drug consumption, respectively) is in line with figures reported in extended meta-analysis of SLIT trials (9). Moreover, these results were made more robust by the observations that the number of well days, a clinical measure more related to quality of life, was increased in treated *versus* control subjects.

Taken together, our data demonstrate that a pre-co-seasonal course of SLIT with an invariable dose of a modified allergen in patients with allergic rhinitis is clinically efficacious and can affect biological parameters which are expected to be associated with the systemic down-regulation of the Th2 allergen-specific immune response and with the tolerance to subsequent allergen exposure.

Notably, birch allergic patients who did not undergo any immunotherapy, when evaluated at the end of the pollen season, displayed an average 54.0% increase in IL-10 expression as compared to the pre-seasonal evaluation, which was made in winter. Although not significant, this result suggests that natural exposure to the allergen can sustain a measurable, although overall insufficient, IL-10 production by regulatory T cells. This observation is reminiscent of a different model of natural exposure, namely IL-10 hyper-production (and associated T cell anergy) in hyperimmune subjects who received multiple bee stings (3).

The majority of studies on SCIT have described a decreased T cell responsiveness to allergen, whereas contradictory results have been reported in SLIT treated patients (15-16, 25-26). However, biologic parameters that can be referred to the tolerogenic effects of modified allergens should not be directly

compared with those obtained with native allergens. In fact, a different pharmacokinetic profile of these two kinds of extracts was reported in experimental models focused on the absorption of recombinant allergens at the oral mucosa level (10-11). These data suggest that the allergoid may reach the intestinal tissue in an un-degraded form and thereby more efficiently amplify the induction of immunological tolerance by acting with local immunocompetent cells in Peyer's patches and mesenteric lymph nodes. Further studies are needed to clarify this issue.

The cytokine data we report here are in agreement with results by Valovirta's group, showing the increase of the IL-10 cytokine in children undergoing SLIT for birch allergy (17). In contrast, no systemic changes were seen in cytokines during build-up or early maintenance phase by Dehlink et al. (27), who used different methods for IL-10 detection and different doses of immunotherapy (below a total of 10 μg of major allergen). Our data are also in agreement with Bohle et al. (28), who reported a reduced proliferative response to allergens and an enhanced IL-10 mRNA expression after 4 weeks of SLIT. Furthermore, these Authors, who also measured Th1 cytokines, found that at 52-weeks of SLIT allergen-induced proliferation remained reduced at the same time as signs of immune deviation emerged. According to these data, an inverse correlation between IL-10 production and allergen proliferation may be limited to the initial phase of SLIT.

We did not find any significant correlation between the measured biological parameters and the clinical scores, and we suggest that this may be due to the small sample size. Moreover, we used in the present work a reductionistic approach focused on the two cytokines which have been more consistently associated to the T regulatory immune response. More comprehensive investigations including Treg, Th17, Th1 and Th2 axis cytokines are needed to clarify the interplay between allergen specific T cells endowed with a regulatory function and effector Th2 cells (4).

The simplified IT protocol we used, which is devoid of any induction phase and require the intake of the same dose of vaccine from the first day of treatment, confirmed its safety and efficacy, in agreement with a recent observation by our group

(24) and with previous report by Rossi et al. (29-30). In particular, treated subjects did not suffer even those minor local problems in the oral mucosa which are reported by the majority of SLIT treated patients (9). This can likely be explained by the usage of a modified, rather than a native, allergen extract. We are convinced that this high tolerability, associating the same efficacy level of immunotherapy with unmodified native allergens, allows to obtain a better compliance to treatment, which is a potentially critical issue in clinical practice, particularly at the beginning of sublingual immunotherapy courses.

The amount of Bet v 1 major allergen, as extrapolated from the amount of native allergen which was used for the preparation of allergoid, was 3.5 μg per dose, and reached a cumulative value of 315 μg. This figure is in the range described in controlled clinical trials with birch allergen extracts (31-33).

In conclusion, the proliferation of allergen-stimulated T lymphocytes was decreased and the transcription of IL-10 was augmented after a pre-co-seasonal 6-month course of SLIT with a modified allergen in birch allergic individuals. Allergen proliferation assays, which are technologically simple although not suitable to high-throughput clinical laboratory, may provide an effective immunological marker of successful immune regulation in the follow-up of allergic patients undergoing immunotherapy.

ACKNOWLEDGEMENTS

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