

SUBLINGUAL IMMUNOTHERAPY AFFECTS SPECIFIC ANTIBODY AND TGF- β SERUM LEVELS IN PATIENTS WITH ALLERGIC RHINITIS

G. CIPRANDI, M. DE AMICI¹, M.A. TOSCA², A. PISTORIO³ and G.L. MARSEGLIA¹

Semeiotica e Metodologia Medica I, DIMI, University of Genoa, Azienda Ospedaliera Universitaria San Martino, Genoa; ¹Clinica Pediatrica, Foundation IRCCS San Matteo, University of Pavia, Pavia; ²Pneumology and Allergology, IRCCS G. Gaslini, Genoa; ³Epidemiology and Statistics Unit, IRCCS G. Gaslini, Genoa, Italy

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Allergic rhinitis (AR) is characterized by Th2 polarized immune response, such as increased IL-4 and reduced IFN- γ production. Sublingual allergen-specific immunotherapy (SLIT) induces several immunological changes, most of which are still little known. The aim of this study is firstly to investigate the changes of allergen-specific IgE, IgG, IgG4, and IgA serum levels after SLIT. Secondly, this study aims at relating immunoglobulin (Ig) values with some Th1-, Th2-, and Treg-dependent cytokines. Twenty-three patients with pollen-induced AR were enrolled, and they assumed two pre-seasonal SLIT courses for 2 years. Serum allergen-specific IgE, IgG, IgG4 and IgA levels were determined by ELISA method at baseline and after each SLIT course. Serum IL-4, IFN- γ , IL-10, and TGF- β levels were also assessed. Allergen-specific IgE, IgG, IgG4, and IgA serum levels significantly increased after SLIT. Serum TGF- β significantly increased after SLIT. There was a significant correlation between IgA and TGF- β , both after the first and the second SLIT course. In conclusion, the present study provides the first evidence that pollen SLIT significantly affects Ig production, mainly concerning IgA; and IgA increase is related with TGF- β production. Moreover, this is the first study that measured Ig classes by using a quantitative method.

AR management includes patient education, allergen avoidance, drug therapy, and, when appropriate, allergen-specific immunotherapy (SIT) (1). SIT is the practice of administering gradually increasing doses of the causal allergen in order to reduce allergic symptoms resulting from exposure and the need for medications. These effects depend on achieving clinical tolerance of the causal allergen. For these reasons SIT is also called “anti-allergic vaccine”. There is evidence that SIT is effective in patients with allergic rhinitis and mild forms of

asthma, as stated in a WHO position paper (2).

SIT is classically based on the subcutaneous administration of allergen extracts (SCIT), but this route of administration has shown a risk of serious adverse reactions; the sublingual (SLIT) route of immunotherapy was therefore investigated and developed as an alternative. The rationale of SLIT is that oral administration of high-dose allergen may reduce and prevent IgE responses. Methodologically, the extract is kept under the tongue for a few minutes and then swallowed: the so-called sublingual-

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Mailing address: Giorgio Ciprandi, M.D.
Semeiotica e Metodologia Medica I,
Viale Benedetto XV 6,
16132 Genoa, Italy
Tel: ++39 10 35338120
Fax: ++39 10 5556696
e-mail: gio.cip@libero.it

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swallow.

Different immunological cellular changes, putatively underlying clinical tolerance to the causal allergen, have been reported following SCIT. These include reduction of the inflammatory cells, such as eosinophils and mast cells, in allergic mucosal tissues (3) as a consequence of enhanced IFN- γ (4), suggesting a preferential deviation of Th2 in favour of Th1 responses. With regard to humoral changes, initially an increase was noted in allergen-specific IgG, mainly of the IgG4 subclass (5), and a modest reduction of specific IgE after SCIT (6). Furthermore, a number of recent studies have indicated that regulatory T cells (Tregs) play an important role in controlling the Th2-biased responses after immunotherapy (7). Indeed, a role for regulatory cells has been shown in patients treated with SCIT for grasses and house dust mites (7). On the basis of these findings, it has been pointed out that successful immunotherapy modifies the T cell response to allergen through the induction of regulatory mechanisms that appears to be defective in untreated allergic patients (8). Indeed, the normal healthy response to allergen is characterized by suppression of allergen-specific responses by T regulatory cells. This issue appears crucial, as a functional defect of T regulatory cells has been provided by an interesting experimental study on allergic subjects (9). Production of inhibitory cytokines, such as IL-10 and TGF- β , is thought to be relevant for the function of T regulatory cells. IL-10 exerts numerous anti-inflammatory activities, including down-regulation of both Th1 and Th2 cytokine production and anergy of effector T cells, and also increases allergen-specific IgG4 production, inhibiting IgE synthesis. Also TGF- β has multiple regulatory effects on T cell proliferation, antigen presentation and expression of co-stimulatory proteins (10). In addition, TGF- β drives the production of IgA (11). Successful subcutaneous immunotherapy is followed by increased production of both IL-10 and TGF- β . Furthermore, increased IgG4 and IgA production has been demonstrated to be associated with IL-10 and TGF- β production (11).

Therefore, the different response to allergens between normal subjects and allergic patients consists in the class of produced allergen-specific antibodies: IgG and IgA in normal subjects, IgE in

allergic patients. SCIT may hence restore a normal T regulatory cell function that consists in inducing an allergen-specific clinical tolerance.

A very recent review also reports that SLIT provides induction of allergen-specific tolerance, as documented by restored allergen-specific IL-10 and IFN- γ production and increased production of IgG4 using extracts of mites or of cypress, but not of IgA using mite extracts. The increase in IgG4 seems to be allergen-dose dependent (1). However, no study has investigated the antibody production after pollen-SLIT comparing the results with some serum Th1-, Th2-, and Tregs-dependent cytokines.

Therefore, the aim of this study is to investigate the effectiveness of two pre-seasonal SLIT courses in affecting allergen-specific IgE, IgG (total and IgG4), and IgA production with relation to serum IL-4, IFN- γ , IL-10, and TGF- β .

MATERIALS AND METHODS

Patients

Twenty-three monosensitized patients (12 males and 11 females) with AR due to pollen allergy were included in the study; 13 of them were allergic only to *Betula alba*, 7 only to *Parietaria judaica*, and 3 only to *Graminaceae*. Their mean age was 38.7 years (SD: 14.3). In order to be enrolled in the study, patients were required to have AR due to pollen allergy. Patients with documented allergy to non-pollen allergens were excluded. According to validated criteria (12), in order to make a diagnosis of AR, a history of nasal symptoms during at least two previous pollen seasons and concordant positive results to skin prick test were considered as sufficient.

Patients' blood samples for assessing serum levels were performed in a standardized manner and collected before initiating SLIT outside the pollen season (baseline, T0), immediately after the end of the first SLIT pre-seasonal course (T1) which lasted 3 months, three months after the end of the first course (T2), and three months after the second SLIT course (T3). Sera were separated from all blood samples within four hours and stored at -30°C until used. Immediately prior to use, serum samples were thawed and centrifuged to remove aggregates. The study was conducted with the approval of the local Ethics Committee (EC) and performed with the written informed consent of all participants.

Specific Immunotherapy

Subjects undergoing SLIT received either a *Betula alba* pollen allergen extract (Anallergo, Florence, Italy;

13 cases, cumulative dose was 60 µg), *Parietaria judaica* (7 cases, cumulative dose was 24 µg), or a mix of three *Graminaceae* (3 cases, cumulative dose was 24 µg) for at least three months before two consecutive pollen seasons, as pre-seasonal immunotherapy. The dose during the maintenance phase was five drops 3 times per week.

Specific IgE antibodies

Allergen-specific IgE ImmunoCAP (Phadia) was measured using the Fluorezyme-immunoassay (FEIA). The allergen of interest, covalently coupled to ImmunoCAP, reacts with the specific IgE in the patient serum specimen. ImmunoCAP Specific IgE Calibrators are used for determination of specific IgE antibodies and values are expressed in kU/l. Values ≥ 0.35 kU/l represent a progressive increase in the relative concentration of allergen-specific antibodies. The results < 0.35 kU/l represent absence or undetectable levels of allergen-specific antibodies.

Specific IgG, IgG4 antibodies

Determination of allergen-specific IgG4 and total IgG antibodies was carried out on all sera against the causal allergens: *Betula alba*, grasses, and *Parietaria judaica* in a single laboratory session (Phadia AB, Uppsala, Sweden) using an IgG or IgG4 ImmunoCAP FEIA assay described previously (13). ImmunoCAP Specific IgG (IgG4) Calibrator Strip was used for determination of total IgG (IgG4) and values were expressed in mg/L (mg/l). The detection limit for IgG was < 0.02 mg_A/L. The limit of quantitation for antigen specific IgG4 antibodies is 0.07 µg/L.

Specific IgA antibodies

Specific IgA ImmunoCAP (Phadia) was experimentally used for research purposes only (measured for those pollens, using the Fluorezymeimmunoassay (FEIA) as previously reported) (13). The calibrators are traceable to the International Reference Preparation (IRP) 67/86 for Human Serum Immunoglobulins A, G and M from the World Health Organization (WHO). The measuring range for a diluted sample is 1.0-100 mg_A/l (where A represents antigen-specific antibodies). The detection limit is < 0.01 mg_A/l.

Cytokine Immunoassays

IL-4

The Human IL-4 Immunoassay (R&D Systems) employs the quantitative sandwich enzyme immunoassay technique and was performed according to the manufacturer's instructions and expressed as pg/ml. The minimum detectable dose was typically less than 10 pg/ml. Reference values for IL-4 were 0-31.2 pg/ml.

IFN-γ

This assay (R&D Systems) employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for IFN-γ has been pre-coated onto a microplate. This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human IFN-γ produced at R&D system. The Minimum detectable dose (MDD) of IFN-γ is typically less than 8.0 pg/ml. Reference values for IFN-γ were 0-15.6 pg/ml.

IL-10

The Human IL-10 Immunoassay (R&D Systems) employs the quantitative sandwich enzyme immunoassay technique and was performed according to the manufacturer's instructions and expressed as pg/ml. The minimum detectable dose was typically less than 3.9 pg/ml. Reference values for IL-10 were 0-7.8 pg/ml.

TGF-β 1

This assay (R&D Systems) employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TGF-β 1 has been pre-coated onto a microplate. Standards, controls and samples are pipetted into the wells and any TGF-β 1 level present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β 1 is added to the wells to sandwich the TGF-β 1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of TGF-β 1 bound in the initial step. The colour development is stopped and the intensity of the colour is measured (pg/ml). This immunoassay is calibrated against a highly purified CHO cell-expressed recombinant human TGF-β 1 produced at R&D system. The range of TGF-β 1 measured was from 1.7 – 15.4 pg/ml. The mean was 4.61 pg/ml.

Statistical analysis

Descriptive statistics were calculated and reported in terms of frequencies and percentages for qualitative variables and in terms of medians with first and third quartiles (q1 and q3) for quantitative data.

Analysis of the levels of the specific Immunoglobulins and cytokines at different time points was performed by means of the non-parametric Analysis of Variance (Friedman's test); Wilcoxon's test for paired data with the Bonferroni's correction was used for *post-hoc* comparisons (p_B). The correlation between quantitative parameters (eg.: correlation between TGF-β and specific IgA levels) was evaluated by the non-parametric Spearman's correlation coefficient (r_s). For the purpose of this analysis, correlation coefficients ≥ 0.8 were considered very strong, from 0.6 to 0.79 were considered strong, from 0.4 to 0.59 were considered moderate, from

0.2 to 0.39 were considered weak and less than 0.2 were considered very weak (14).

All statistical tests were two sided; a P value of less than 0.05 was considered as statistically significant. The statistical package "Statistica" (StatSoft Corp., Tulsa, OK) was used for all the analyses.

RESULTS

Twenty-three patients with AR, 12 males and 11 females, were included in the study. Thirteen of them were allergic only to *Betula alba*, 7 only to *Parietaria judaica*, and 3 only to *Graminaceae*. Their mean age was 38.7 years (SD: 14.3). All patients successfully responded to SLIT on the basis of the reported perception of improvement. As shown in Fig. 1, specific IgE levels significantly changed (Friedman's test; $p=0.022$) during the course of the study; in particular, a statistically significant raise was observed between T0 (median: 12.7; q1-q3: 2.5-27.1) and T2 (median: 18.8; q1-q3: 7.8-40.9) ($p_B=0.006$). IgE levels at T2 were also statistically significant higher with respect to T1 (median: 10.6; q1-q3: 2.3-29.4) ($p_B=0.02$). However, IgE levels tended to decrease at T3 (median: 15.8; q1-q3: 7.2-44.1) with respect to T2.

As shown in Fig. 2A, specific total IgG levels significantly changed (Friedman's test; $p=0.005$); in particular, a statistically significant raise was observed between T0 (median: 3.9; q1-q3: 2.5-5.3) and T1 (median: 4.5; q1-q3: 2.6-6.3) ($p_B=0.038$),

and between T0 and T2 (median: 4.6; q1-q3: 2.7-6.7) ($p_B=0.008$). The IgG levels were not different between T1 and T2 ($p_B=0.26$). Nor was any difference observed between T2 and T3 (median: 4.6; q1-q3: 3.6-7.2). Analogously, specific IgG4 levels tended to increase from T0 (median: 0.2; q1-q3: 0.1-0.7) to T2 (median: 0.3; q1-q3: 0.1-0.7) and T3 (median: 0.4; q1-q3: 0.3-0.6). The statistical significance of this increment was weak (Friedman's test; $p=0.021$).

As shown in Fig. 3, specific IgA levels significantly changed (Friedman's test; $p=0.0002$) during the course of the study; in particular a statistically significant raise was observed between

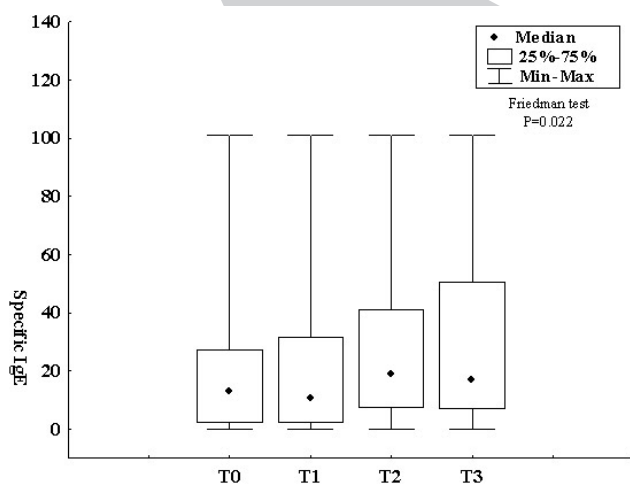


Fig. 1. Specific IgE levels at different time points of SLIT treatment. The data are expressed as kU/L.

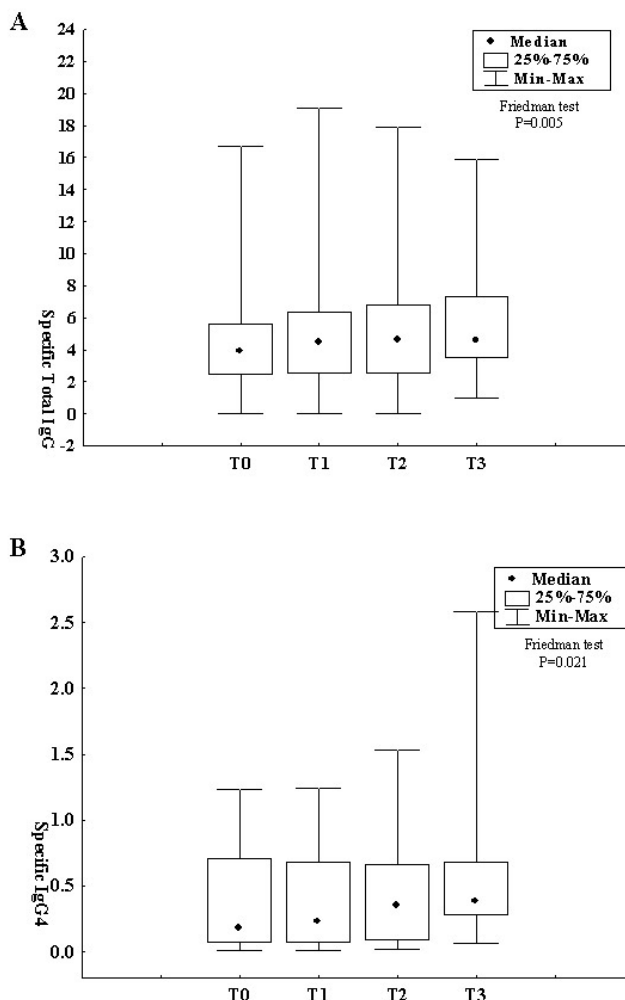


Fig. 2. Specific Total IgG (panel A) and Specific IgG4 levels (panel B) at different time points of SLIT treatment. Data are expressed as mg/L.

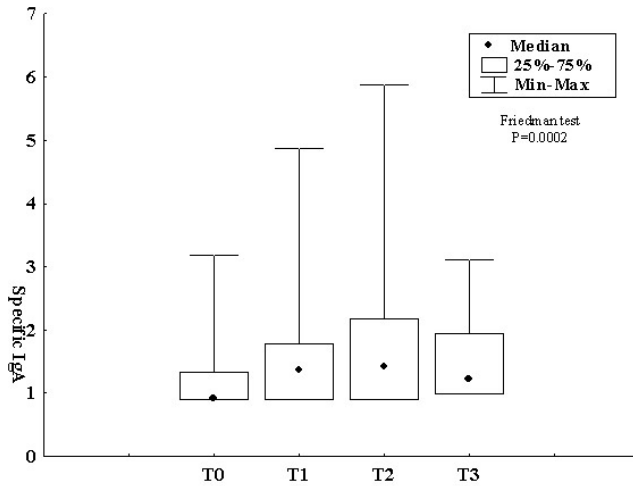


Fig. 3. Specific IgA levels at different time points of SLIT treatment. Data are expressed as mg/L.

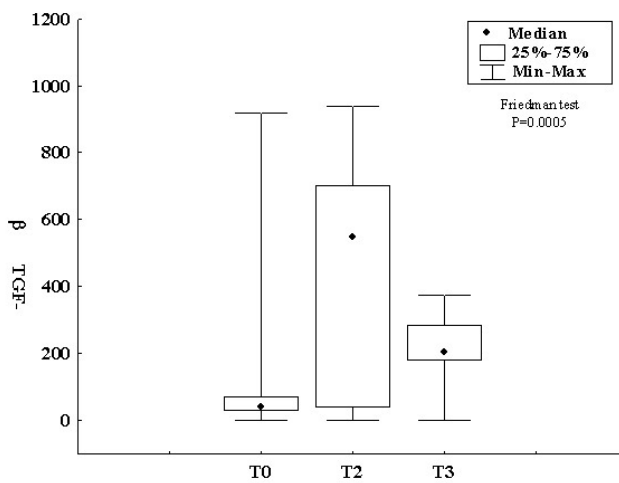


Fig. 4. TGF- β levels at different time points of SLIT treatment. Data are expressed as pg/mL.

T0 (median: 0.9; q1-q3: 0.9-1.3) and T1 (median: 1.4; q1-q3: 0.9-1.8) ($p_B=0.003$) and between T0 and T2 (median: 1.4; q1-q3: 0.9-2.1) ($p_B=0.003$). In addition, IgA levels were slightly lower at T3 (median: 1.2; q1-q3: 1.1-1.9) with respect to T2, but still remaining higher with respect to T0 ($p_B=0.007$).

As shown in Fig. 4, serum TGF- β levels significantly changed during follow-up (Friedman's test; $p=0.0005$); in particular, a statistically significant increase was observed between T0

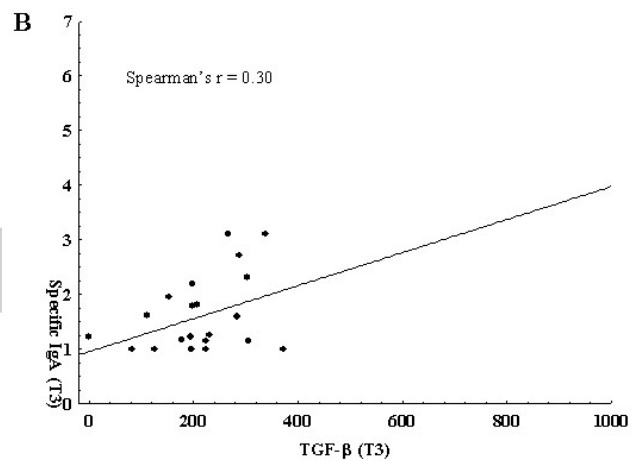
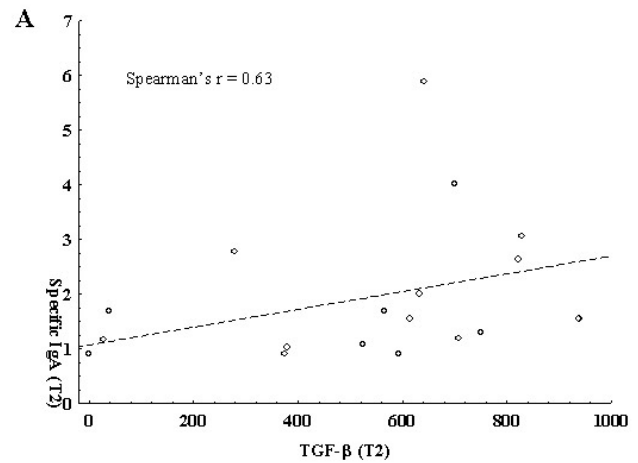


Fig. 5. Correlation between IgA levels and TGF- β at T2 (panel A) and at T3 (panel B).

(median: 39.4; q1-q3: 31.9-68.8) and T2 (median: 545.7; q1-q3: 41.3-686.3) ($p_B=0.006$). The TGF- β levels decreased at T3 (median: 203.4; q1-q3: 183.5-280.0) with respect to T2 ($p_B=0.02$), but were still higher with respect to T0 ($p_B=0.02$).

Serum IL4 levels tended to decrease from T0 (median: 8.5; q1-q3: 0.1-15.2) to T1 (median: 5.7; q1-q3: 0.1-17.6) and to T2 (median: 0.5; q1-q3: 0.1-11.8), but this change was not statistically significant (Friedman's test; $p=0.43$). Serum IFN- γ levels tended to increase from T0 (median: 6.8; q1-q3: 1.6-11.9) to T1 (median: 10.8; q1-q3: 6.1-17.1) and to T2 (median: 12.4; q1-q3: 4.3-21.8),

but this increment was not statistically significant (Friedman's test; $p=0.15$). Serum IL-10 levels did not change substantially from T0 (median: 2.2; q1-q3: 0.1-15.9) to T1 (median: 0.8; q1-q3: 0.1-6.8) and to T2 (median: 3.1; q1-q3: 0.1-18.3), but these changes were not statistically significant (Friedman's test; $p=0.32$). A strong correlation was observed between specific IgA levels and TFG- β at T2 (Fig. 5A) ($r_s=0.63$); at T3 (Fig. 5B) the correlation became weak ($r_s=0.30$), whereas there was no significant relationship between T0 and T1 ($r_s=0.08$). On the other hand, no correlation was observed among all the remaining parameters.

DISCUSSION

Allergic rhinitis is characterized by a Th2-polarized inflammation. Th2-derived cytokines, such as IL-4 and IL-13, are the primary pathogenic factors in inducing, maintaining, and amplifying inflammatory allergic inflammation. IL-4 and IL-13 orchestrate allergic inflammation promoting IgE synthesis, up-regulating adhesion molecules selective for eosinophil recruitment, and causing increased mucus production and airway hyperreactivity. On the other hand, there is accumulating evidence that Th1-related cytokines, such as IFN- γ and IL12, may suppress and counteract this Th2 response and vice-versa, as there is functional dichotomy between Th1 and Th2 cells (1).

SIT is highly effective in the treatment of AR, as it is the only treatment which leads to a life-long tolerance against previously disease-causing allergens, resulting from the restoration of normal immunity (1).

Recently, it was demonstrated that SLIT is a safe and effective route of administration of allergen extracts in allergic patients. However, its mechanism of action is still poorly understood, as there are few data concerning the immunologic changes induced by SLIT (1). Few studies have shown a relevant effect on the IgG4 response after SLIT, thus it could seem that SLIT does not consistently reduce the IgE production and increase the IgG4 synthesis as the results of these studies are conflicting (15-19). On the contrary, a decrease of serum IL-13 was demonstrated after SLIT therapy (20) as well as an increase of Th1-related cytokines (21). Moreover, it

has been reported that T-cell proliferative response was reduced by SLIT (22).

The discrepancies in these studies may depend on several methodological factors, including the type of allergen, the duration of SLIT, the age of patients and the AR duration, the dose of allergen extracts used in *in vitro* experiments, the kinetic of incubation, and the methods used to assess cytokines. For example, the study of Dehlink et al. evaluated cytokines and Ig response (including IgE, IgG and IgA) 2 and 8 weeks after the initiation of SLIT: periods perhaps too brief to detect immunologic changes considering the type of allergen extract, such as house dust mites (23).

Yet other studies in animal models have provided positive confirmation of the fact that the oral route is tolerogenic and can reverse the Th2 polarization (24-26). Moreover, a very recent study indirectly supports the relevance of the Th2-Th1 shift as SLIT increases the expression of both IL-18 and SLAM (20). IL-18 is a macrophage-derived cytokine originally found as a factor inducing IFN- γ production from Th1 cells (26). SLAM (signalling lymphocytic activation molecule) is a self-ligand, trans-membrane, cell surface glycoprotein, and its activation promotes T-cell proliferation, and IFN- γ production. It is preferentially expressed in Th1 cells over Th2 cells (27). Therefore, Th1 polarization represents an important change in the immune response after SLIT, as recently confirmed by one study that provided evidence that successful SLIT is associated with an early IFN- γ production (28). Moreover, the production IFN- γ , that is specifically defective in allergic patients (29), restores after SLIT, showing also a carry-over mechanism (30).

Consequently, SLIT re-directs the immune response toward a Th1 response and restores an efficient T regulatory function. As a consequence of these phenomena, a new balance among cytokines occurs. These cytokines, namely IFN- γ , IL-10, and TGF- β , are crucial to re-direct antibody synthesis toward IgG and IgA classes instead of the IgE class.

The present study firstly provides evidence that two pre-seasonal SLIT courses using pollen extracts significantly affect allergen-specific Ig production. In particular, serum IgE levels initially increase during the first course, then tend to diminish: this result confirms the positive studies. Total IgG and IgG4,

as well as IgA, significantly increase during the first course. However, the increase is more evident and relevant for allergen-specific IgA.

Likewise, TGF- β significantly increases after SLIT courses, even though more clearly during the first year, thus supporting the Ig kinetics. Moreover, the significant correlation between IgA changes and TGF- β increase further confirms this response pattern. Unfortunately, the assessment of serum IL-4, IFN- γ , and IL-10 cytokines is not relevant; it might depend on the relatively low sensitivity of the serum measurement as previously reported (28).

The most relevant and original finding of this study is therefore represented by the fact that it provides the first evidence showing that also pollen SLIT is capable of significantly increasing IgA production; this phenomenon is also correlated with TGF- β production. This issue is particularly relevant as it demonstrates that the pattern of allergen-specific response shifts from IgE to IgG and mainly IgA synthesis. This fact supports the occurrence of a restored tolerance to the causal allergen that is translated in the appearance of functioning T regulatory cells, as demonstrated by increased TGF- β values.

In conclusion, the present study shows that SLIT is capable of inducing immunologic hyporeactivity directed to environmental antigens, in allergic patients presenting with AR due to pollen sensitisation. Indeed, SLIT induced systemic immunologic changes very rapidly. Therefore, these results may be considered clinically relevant proof of SLIT treatment inducing a quick change of Ig response patterns. These preliminary findings need to be confirmed by further studies.

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