EARLY CYTOKINE MODULATION AFTER THE RAPID INDUCTION PHASE OF SUBLINGUAL IMMUNOTHERAPY WITH MITE MONOMERIC ALLERGOIDS

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The influence of different treatment schedules of sublingual immunotherapy (SLIT) in activating IL-10-producing T-cells, crucial in inducing allergen-specific tolerance, is not completely understood. The present work was designed to evaluate allergen driven interleukin release by mononuclear cells in the early phase of SLIT, after application of different induction schemes. Twenty mite-allergic patients were enrolled, 10 (group A) treated with a traditional 98 day induction scheme and 10 (group B) with a 16 day scheme with monomeric allergoid vaccine. At the end of the induction phase, the cumulative doses taken by group A and group B patients were equivalent to 50.5 and 50.3 μg of mite group 1 allergens, respectively. The release of Th1-, Th2- and Treg-related interleukins was assessed in culture supernatants of 5 μg/ml Der-p1-stimulated mononuclear cells, isolated before and after the induction phases. No relevant treatment-related side effects were observed. Interleukin release was similar in the two groups at the enrolment. Non-stimulated and Der p 1 stimulated release of studied cytokines was similar in the two groups at enrolment. Der p 1 stimulation significantly increased IL-10 release ($p<0.0002$) after treatment in group B patients, and this effect was higher ($p=0.05$) compared to group A patients. Furthermore, at the end of SLIT induction TNF-alpha, IL-4 and IFN-gamma production were reduced in group B patients ($p<0.05$, $p=0.062$ and $p=0.060$, respectively). The rapid induction scheme of sublingual immunotherapy induces an early immune suppression more effectively than the slower one. The rapid induction scheme should be the preferential way to start sublingual immunotherapy, particularly when monomeric allergoids are utilized.

Since its introduction almost a century ago, allergen-specific immunotherapy (SIT) has been shown to be an effective therapeutic tool for the treatment of patients with severe allergic rhinitis and/or asthma (1-2). The association of SIT with uncommon, but severe, or even fatal systemic reactions (1, 3-4) has stimulated investigators to explore safer therapeutic modalities. Chemical modification of allergens by potassium cyanate (KCNO) to reduce their IgE-binding capacity

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and obtain monomeric allergoids (5) created the basis for the development of safer vaccines to be administered by alternative non-injectable route, the most effective being the sublingual one, introduced into clinical practice during the 1990s. Sublingual immunotherapy (SLIT) has been found to induce similar immune modifications as the subcutaneous route both in animal models (6) and in humans, in particular a switch from Th2 to Th1 cytokine profile (7-8) and a progressive increase in IL-10-producing cells, with a consequent reduction of allergen-induced $CD4^+$ cell proliferation (9).

Recently, in consideration of the high level of safety provided by monomeric allergoid vaccines, various investigators have performed treatment schedules based on a reduction of the induction phase, obtaining significant clinical benefits without significant side-effects (10). However, there are no comparative studies that verified the difference in immunological changes induced by the classical and the rapid schemes of the induction phase with SLIT. On the contrary, McHugh et al. showed that rush subcutaneous hymenoptera venom immunotherapy (VIT) induced an earlier and more effective immune modulation than the traditional scheme of treatment (11). Furthermore, a recent study on the efficacy of the ultra-rush venom immunotherapy showed that VIT modulated the immune response as soon as the maintenance dose of 111.21 μg was reached at day 1 of treatment (12).

The present work was designed to evaluate whether also with SLIT, a rapid dose build-up phase of treatment could induce a more rapid modulation of the immune system than the traditional induction schedule (13-14). The study included twenty patients allergic to *Dermatophagoides pteronyssinus* (Dp). They were divided into two groups and treated with mite monomeric allergoid (Lofarma, Milan, Italy) according to: 1) a traditional (98 days) or 2) a rapid dose build-up scheme (16 days). Before, and at the end of the induction phases, the *in vitro* Der-p1 driven modulation of several cytokines characteristic of the Th1, Th2 and Treg pattern (IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α) was evaluated.

MATERIALS AND METHODS

Twenty patients with mite allergy, high level mite-specific serum IgE and a history of severe persistent allergic rhinitis and mild persistent asthma (9 patients) were included in the study. The patients had never been treated with specific immunotherapy. Their demographic and clinical data are presented in Table I. Skin prick tests for other inhalant allergens (pollens, moulds, cockroaches and animal dander) were negative. Asthma was treated by inhaled steroids and long acting beta2 agonists according to the GINA (Global Initiative for Asthma, 2006) guidelines, which allowed to reach an optimal asthma control before starting the specific immunotherapy. Intranasal fluticasone was prescribed for the treatment of rhinitis. The patients were allowed to take beta2 agonists and antihistamines as needed. The study was performed with the approval of the Ethics Committee of the "G. d'Annunzio" University of Chieti, Italy, after the patients' written informed consent. The study design is shown in Fig. 1.

Specific immunotherapy

A commercial SLIT treatment with a mite monomeric allergoid (LAIS, a 50% mixture of Dp and Df extracts, Lofarma SpA, Milan, Italy) was prescribed to all selected patients. The monomeric allergoid was obtained by carbamilation with potassium cyanate at alkaline pH, a reaction that leads to a substantial substitution of the ε-amino groups of lysine residues. This product, standardized for allergenic potency by EAST-inhibition in comparison with an in-house reference preparation and titrated in allergenic units (AU), is formulated in orosoluble tablets and administered sublingually.

Group A followed a traditional induction phase, with increasing doses from 25 to 3000 AU to be reached in 98 days, with a three times/week administration of the allergen extracts, according to the manufacturer's conventional protocol. Group B followed a rapid induction phase that allowed to reach the maintenance phase in 16 days with bid administration of allergen extracts. The cumulative dose reached was 18,525 UA equivalent to 50.5 μg of group 1 mite allergen (50% of Der-p1+ 50% of Der-f1) for group A and 18,425 UA, equivalent to 50.3 μg of group 1 mite allergen for group B (Table II).

Peripheral blood mononuclear cell culture and cytokine quantification

Venous blood samples were collected from each patient before and at the end of the build-up phase of the treatment with vacutainer tubes containing EDTA as an anticoagulant. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood within a few hours from sampling by Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation and resuspended in RPMI-1640 medium (Sigma-Aldrich, Gallarate, MI,

Italy) containing L-glutamine and 25 mM HEPES buffer (Sigma-Aldrich) supplemented with 10% fetal calf serum (Euroclone, Milan, Italy) and penicillin (100 UI/ml)-streptomycin (100 µg/ml) (Sigma-Aldrich). The final concentration of cell cultures was adjusted with complete culture medium to 1×10^6 mononuclear cells/ml. For each patient, PBMC (1×10^6 cells/ml) were cultured in medium alone or stimulated with 20 µg/ml PHA-M (Sigma), or 5 µg/ml Der-p1 monomeric allergoid (Lofarma, Milan, Italy), or 20 µg/ml PHA+5 µg/ml Der p 1, and incubated at 37°C in 5% CO_2 atmosphere. After five days, the cell suspensions were transferred to tubes and centrifuged at 1500 rpm for 10 min. The supernatants were collected, aliquoted and stored at −70°C for cytokine determination. All experiments were performed in triplicate.

Cytokine quantification

Th1, Th2 and Tregs cytokines in cell culture conditioned supernatants, including IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α, were quantified simultaneously using a human cytokine cytometric bead array kit (BD, San Diego, CA, USA). This assay kit provides a mixture of six microbead populations with distinct fluorescent intensities, pre-coated with capture antibodies specific for each cytokine. Assays were performed according to the manufacturer's instructions. Individual cytokine concentrations were indicated by their fluorescent intensities detected by FACSCalibur flow cytometer (BD, San Diego, CA, USA) and were computed using the standard reference curve of CELLQUEST and CBA software (BD, San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed comparing the mean percentage variation [(value after stimulation - value before stimulation)/value before stimulation X 100] after treatment in respect to before therapy, both of all treated patients (to evaluate the global effectiveness of treatment) and for each specific protocol to evaluate significance of differences between rapid versus classical treatment regimen. Since the sample size, i.e. the number of patients enrolled in the study, is relatively small, non-parametric statistical procedure (Mann-Whitney test) was applied. Statistical analysis was performed using SPSS® Advanced Statistical 11.0 software (SPSS Inc, Chicago, Illinois, USA). In all analyses, the level of significance was set at $p < 0.05$.

RESULTS

All patients completed the induction phase of SLIT without significant side effects other than bearable oral itching in 3 patients treated with classical dose build-up scheme and in 2 patients treated with the rapid induction protocol. Group A patients reached the cumulative dose of 50.5 µg of group 1 mite allergen within 98 days, and group B 50.3 µg of group 1 mite allergen within 16 days. Two patients of group A and four of group B reported episodic use of short acting beta agonists as rescue medication for asthma. Finally, no patients required changes in the basic pharmacological treatment of asthma or in the SLIT administration scheme.

Table I. *Demographic and clinical data of the patients included in the study.*

	Group A	Group B
Number of subjects	10	10
Male/female	3/7	6/4
Age (years)	28 (±6)	24 (±5)
Rhinitis	6	5
Asthma and rhinitis	4	5
Mite IgE (KU/l *)	7.3 (±3)	12.1 (±4.3)

*Characteristics are described for the included subjects according to the two considered groups. Data are expressed as means (±SD); * CAP, Phadia, Sweden*

Table II. *Posological schemes for the build-up phase treatment.*

Group A		Group B	
Week	Dose/week	Day	Dose/day
1	75 AU	1-3	75 AU
2	150 AU	4-6	300 AU
3	300 AU	7	600 AU
4	600 AU	8-10	900 AU
5-6	900 AU	11	2,000 AU
7-8	1,800 AU	12-14	2,000 AU
9-14	3,000 AU	15-16	3,000 AU
Total	18,525 AU	Total	18,425 AU

Treatment was carried out with the allergoid extract LAIS (Lofarma SpA, Milan, Italy) AU=Allergen Unit

Table III. *Cytokines release by PBMCs of the two groups of patients before treatment.*

	IFN-γ		*TNF-α*		*IL-10*		*IL-6*		*IL-4*		*IL-2*	
	PHA	PHA+ Der-p1	PHA	PHA+ Der-p1	PHA	PHA+ Der-p1	PHA	PHA+ Der-p1	PHA	PHA+ Der-p1	PHA	PHA+ Der-p1
Group A	**74**	110.7*	**5.7**	6.30	**7.3**	8.19	**29.1**	37.56*	**15.2**	182.0*	**5.1**	8.76*
SD	**68**	93.0	**1.4**	2.49	**4.4**	5.09	**22.1**	26.98	**7.3**	153.3	**1.9**	3.66
Group B	**76**	105.9*	**5.2**	5.75	**9.5**	10.9	**30.1**	39.16*	**12.5**	133.8*	**4.1**	6.17*
SD	**73.1**	83.9	**1.3**	2.36	**5.4**	6.40	**29.4**	35.07	**6.8**	136	**1.1**	1.62

*Der-p1 induced a significant increase in the production of IL-2, IFN-γ, IL-4 and IL-6. No difference was detected in the production of the studied cytokines between the two groups of patients. Cytokines are expressed as ng/mL; *=p<0.05*

Cytokine quantification: all patients

Cytokine production in cultures without PHA was negligible and in many cases below the limit of detection. In PHA stimulated cultures, Der-p1 induced a significant increase in the production of IL-2, IFN-γ, IL-4 and IL-6 in all cultures at baseline (Table III). On the contrary, no significant changes were observed in the production of TNF-α and IL-10. After the SLIT induction phase, we observed in all patients a trend in the IL-2 and IL-6 release by PMBCs similar to that observed before treatment, both in un-stimulated and in Der-p1-treated cultures. In addition, a slight increase in IFN-γ and a reduction in IL-4 and TNF-α release were also detected in Der-p1 stimulated cultures, however, the differences were not significant (data not shown). On the contrary, after treatment, IL-10 release significantly increased (at the limit of significance p=0.05) in Der-p1 stimulated in respect to un-stimulated cultures (Fig. 2).

Cytokine quantification: rapid vs traditional treatment

Before the induction phase, there were no differences in spontaneous or Der-p1 induced cytokine release by PBMCs between group A and B.

After treatment, IL-10 percentage increase (Der-p1-stimulated v/s un-stimulated cultures) was not significant for group A and highly significant (p=0.0002) for group B (Fig. 2).

In group B cell cultures, a significant reduction was also observed in Der-p1-induced release of TNF-α (p<0.05) (Fig. 3). On the contrary, not significant changes were observed in Der-p1-induced release of IL-4 and IFN-γ (p=0.062 and 0.06 respectively) (Fig. 3) and of IL-2 and IL-6 in the two groups and between groups, both in un-stimulated and stimulated cultures.

DISCUSSION

In this study, we demonstrate that SLIT performed with a rapid dose build–up scheme is effective in inducing an earlier and significant immune suppression by activating IL10 release. We compared the allergen-driven release of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α (representative of Th1, Th2 and Treg cytokine pattern) by PBMCs from two groups of mite-sensitive patients suffering from persistent allergic rhinitis, in 9 patients associated with asthma, treated with different SLIT induction schemes. An increase in the Der-p1-driven release of IL-10 was found in both groups, but only in patients treated with the rapid scheme such increase reached a statistical significance (p<0.0002) (Fig. 2). Similar results were obtained with the ultra rush VIT that induced a significant regulatory T cell increase from day 1 of treatment, as soon as the first maintenance dose was reached (12). There are no comparable studies on rapid induction scheme of SLIT, its immune effects having been studied only in

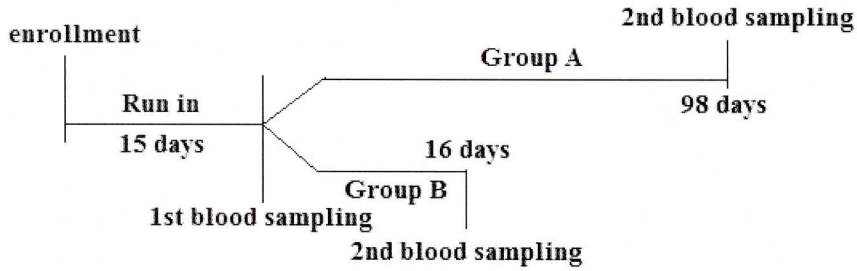

Fig. 1. *Study design. Patients with rhinitis alone or with asthma were enrolled. After a 15 days run in period, patients were randomly assigned to a classic or rapid induction scheme of mite specific sublingual immunotherapy. Blood for analyses was drawn before and after each induction scheme.*

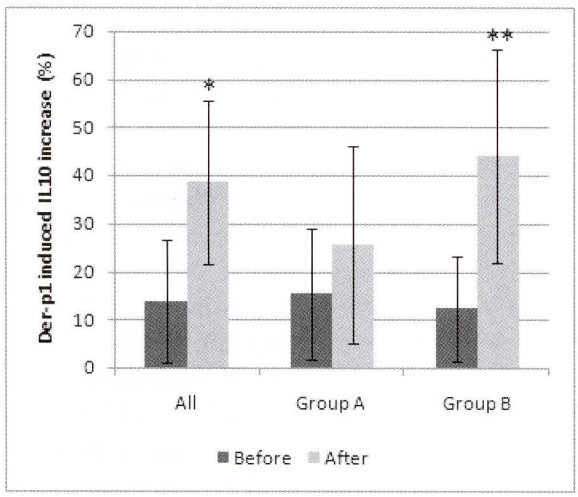

Fig. 2. ***A)*** *Der-p1 induced percent increase of IL-10 in supernatants of culture from all subjects, group A and B. The increase was significant in group B and in all patients. *p=0.05; **p<0.0002.* ***B)*** *The increase in IL-10 production in group B was evident in all studied patients.*

patients treated with the classical 28-day dose build-up schedule (as suggested by the manufacturer of the specific product utilized), and results are contrasting. A recent study demonstrated that SLIT for birch pollen was able to induce IL-10 production during the early phase of treatment (9). On the contrary, other authors did not observe any significant changes in cytokine production (IL-10, IFN-γ, IL-4, TGF-β) in allergen-stimulated cultures during the early phase of treatment (13-14). These different results may be partially explained by the different cell culture protocols applied in the various studies and by the different commercial products utilized. Moreover, in our study the classical induction scheme of treatment (98 days for LAIS) did not induce a significant early increase in IL-10. Indeed, tolerance induction could be driven through several pathways with different rhythms of administrations and doses of allergens.

Later in the course of SLIT, IL-10 production and the number of IL-10-producing cells have been shown to increase by many authors (8, 15-17) and the importance of the up-regulation of this cytokine has been underlined by the fact that the increase in the production of IL-10 has been considered responsible for IT success (18-19). The increase in IL-10 production correlates with the improvement of symptoms and early bronchial involvement in patients with pure allergic rhinitis successfully treated by SLIT for mites (15-16) and for birch pollen (9).

In particular, immunosuppressive IL-10 can induce specific unresponsiveness (anergy) in peripheral T cells (9, 17, 19-21) and was followed by the IT-induced Th2/Th1 switch (22-23), with a decrease in IL-4 and an increase in IFN-γ production in patients undergoing IT. Such changes appear late during the course of SLIT. Cosmi et al. found that the increase in IFN-γ was evident and statistically significant after sixth months of therapy (8). Bohle et al. underlined that in subjects treated by SLIT for birch pollen, a significantly high IFN-γ mRNA expression was found only after 52 weeks of SLIT, concomitant to the decrease in IL10 (9). In our study we found a tendency of an early modulation of IFN-γ and IL4 (Fig. 3A and B), likely as a result of a rapid and strong stimulation of the immune system with the rapid scheme adopted in group B, as happens for rush venom IT. In fact, rush VIT induces a decrease in IL-4 and IL-13 and an increase in IFN-γ

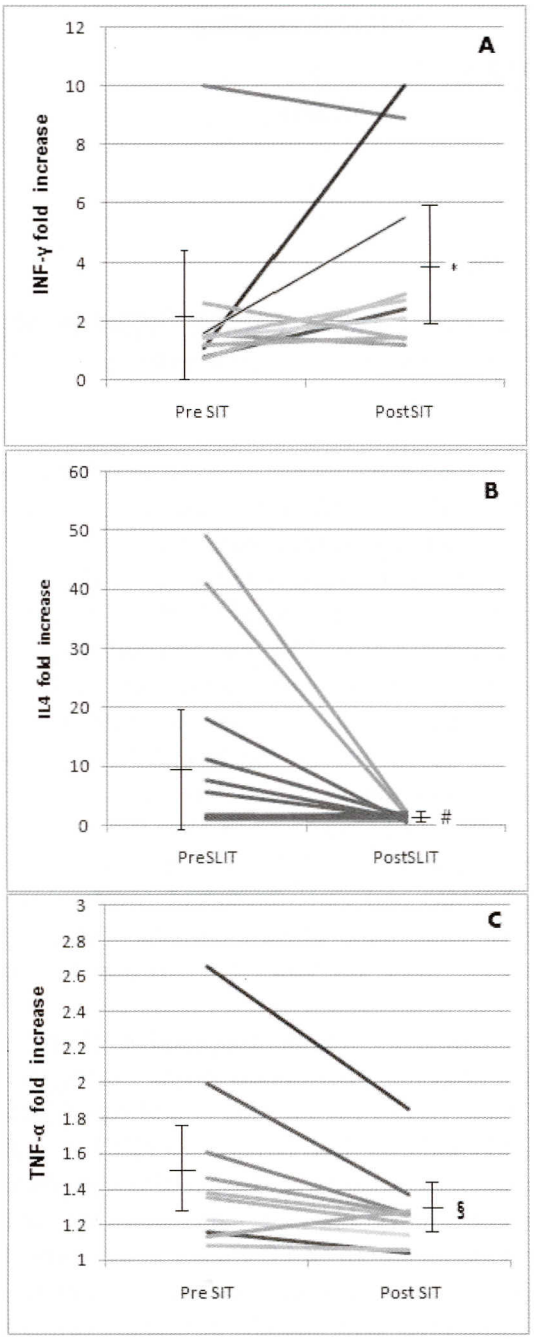

Fig. 3. *Fold increase of IFN-γ (**A**), decrease of IL-4 (**B**) and TNF-α (**C**) in group B patients. Only TNF-α changes was significant. *:p=0.06; #:p=0.062; §:p=0.05*

as early as the first day of treatment, whereas such changes were delayed for 2 months (11-12) during conventional VIT.

There are more difficulties in explaining the reduction in the Der-p1-driven release of TNF-α after SLIT (Fig. 3C). It has been shown that TNF-α tends to increase in severe allergic diseases such as asthma (24) and that the TNF-α/IL-10 ratio is increased in allergic subjects with respect to non-allergic ones, as shown in the tears of patients suffering from allergic conjunctivitis (25). Besides, IL-10 is able to inhibit TNF-α production (26), and such inhibition could be interpreted as an effect of SLIT in preventing the worsening of treated allergic diseases. However, IL-10 being a strong inhibitor of both Th2 and Th1 cytokines, also IFN-γ should be reduced if all cytokine modifications during SLIT are IL-10-dependent, (26-27). It is more likely, in agreement with Bohle et al. (9), that different immune mechanisms are operative during immunotherapy, with a predominant role of IL-10 in the early phases of treatment and with a specific non-reactivity and immune deviation of allergen-specific T cells during the late phases of therapy. Monitoring the production of selected cytokines during the course of SLIT is currently under investigation in our laboratory, in order to verify whether changes in IFN-γ, IL-4 and TNF-α will become predominant relative to IL-10 during SLIT, thus confirming this hypothesis.

As the increase of IL-10 and the Th2/Th1-like cytokine modification seem to be the basis of the therapeutic effectiveness of SLIT, our finding could have a clinical implication: the rapid induction scheme should be the preferential way to start SLIT, in particular when monomeric allergoids are utilized, taking into account their demonstrated safety (10, 28). The allergoid utilized in our study is obtained by carbamilation with potassium cyanate at alkaline pH, a reaction that leads to a substantial substitution of ε-amino groups of lysine residues, and consequently to a strong decrease in the capacity to react with IgE, with the reduction of risk to induce side effects (5). Finally, the rapid induction schedule can be particularly useful for the treatment of patients with pollen allergy who discover their allergy late, near the pollination season, because they can obtain the immune effect of SLIT in a restricted period of time.

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