

SUBLINGUAL SPECIFIC IMMUNOTHERAPY REDUCES PBMC PROLIFERATIONS

D. Fenoglio (1), F. Puppo (1), I. Cirillo (2), A. Vizzaccaro (2),
A. Ferrera (1), M.-A. Tosca (3), G. Marseglia (4), G. Ciprandi (5)

SUMMARY:

Background: Subcutaneous specific immunotherapy has been demonstrated capable of inducing T regulatory response. There is few evidence concerning immunological changes induced by sublingual immunotherapy.

Objective: The aim of this study was to evaluate T cell proliferation in subjects successfully treated with SLIT for HDM.

Methods: PBMCs were isolated from patients after at least 3 years of successful HDM SLIT and from matched untreated allergic and healthy control subjects. After 3 and 6 days of *in vitro* stimulation with PHA, *Candida albicans*, *Dermatophagoides farinae*, grasses, *Parietaria judaica*, and cat, proliferation.

Results: Subjects treated with SLIT showed significant reduction of proliferation induced by *Candida albicans*, *Parietaria*, and grasses in comparison with untreated atopsics ($p=0.0002$, 0.0033 , and 0.009 respectively).

Conclusion: This pilot study confirms reduced T cell proliferation in allergic subjects treated with SLIT.

Key-words: Sublingual immunotherapy - T cell proliferation - Immunologic tolerance.

INTRODUCTION

Specific immunotherapy (SIT) is traditionally based on the subcutaneous administration of allergen extracts to obtain clinical tolerance of the causal allergens in patients with allergic disorder (1). There is evidence that specific immunotherapy is effective in patients with allergic rhinitis and mild form of asthma as stated in a recent WHO position paper (2). Th2 cells play a critical role in the pathogenesis of allergic rhinitis and asthma (3). However, the immunological mechanisms that downmodulate and protect against the development of them are poorly understood. A spectrum of CD4+ T cells, including Th3 cells, Tr1 cells, CD4+CD25+ cells, and NKT cells, might exert a role in regulating allergic reaction (4,5). A better understanding of the role of regulatory cells in allergic disorders may lead to the identification of pathways involved in specific immunotherapy. Recently, a role for regulatory cells has been evidenced in patients treated with SIT for grasses and HDM (6,7). Moreover, the role of IL-10 as early marker of successful immu-

notherapy has been more recently evidenced (8). On the basis of these findings, it has been pointed out that SIT generates regulatory T cells secreting both IL-10 and TGF β and specifically suppress allergen-induced responses (9). This issue appears crucial as a defect of T regulatory cells has been recently provided by an elegant experimental study in allergic subjects (10).

As the traditional subcutaneous route of administration showed the risk of serious adverse reactions, alternative routes of immunotherapy, including oral, sublingual (SLIT), and nasal, have been investigated and developed (11).

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 per few minutes and then swallowed: the so-called sublingual-swallow. A different method may be used: after keeping the allergen under the tongue, it is spat out ("sublingual-spit"). At present, this last method is rarely used.

SLIT efficacy has been evaluated in the WHO position paper (2), in the EAACI/ESPACI document (12), and in the ARIA document (13). All the published studies but three evidenced the clinical efficacy of SLIT both on rhinitis and asthma symptoms (11,14). The efficacy for rhinitis was recently confirmed by meta-analysis Cochrane review (15).

(1) DIMI-CEBR, Università di Genova, Genova, Italy

(2) Navy Hospital, Medicine Dpt., La Spezia,

(3) Istituto Giannina Gaslini, Genova,

(4) Clinica Pediatrica, IRCCS Policlinico San Matteo, Pavia, Italy

(5) Azienda Ospedaliera Universitaria San Martino, Genova, Italy

Although the precise mechanisms by which this oral tolerance is induced are not well understood, the route of allergen processing and presentation remains a critical determinant of the subsequent T-cell modulation (14). Nasal immunotherapy, a variant of local immunotherapy, has been demonstrated to be capable of decreasing allergen-specific T-cell response (16). Very recently, oral specific immunotherapy provided evidence of inducing a form of immunologic tolerance to the allergen (17). However, the immunologic changes induced by SLIT remain still unknown. Thus, our aim was to investigate this issue.

METHODS

Patients

The study was approved by the Review Board of the La Spezia Navy Hospital and performed with the informed consent of all participants. The donor panel consisted of 9 healthy subjects (as control group), 10 allergic subjects, and 9 patients receiving HDM SLIT. Allergy was defined by a positive skin prick test response to *Dermatophagoides pteronyssinus* at least 4 mm greater than that elicited by the negative diluent control (Stallergenes, Milan, Italy). Allergic donors were all symptomatics, with a clear history of allergic rhinitis. Subjects undergoing SLIT had received HDM immunotherapy (Staloral 100 IR, Stallergenes, Milan, Italy) for at least 3 years and were well matched to allergic donors for age, sex, and skin prick test response. Six subjects of untreated group and five subjects of treated group were polysensitized, but the sensitization for other allergens was not cause of symptom occurrence as provided both by their history and negative allergen-specific conjunctival challenge. The study was performed during the winter, such as outside the pollen season.

Clinical evaluation

Patients were assessed for a change in symptoms. The following symptoms were assessed by the patient, answering the questions made by the investigator: nasal obstruction, sneezing, rhinorrhea, and itchy nose. Each symptom was evaluated on the following scale: 0= absent, 1= mild (symptom was present but was not annoying or troublesome), 2= moderate (symptom was frequently troublesome but did not interfere with either normal daily activity or sleep), and 3= severe (symptom was sufficiently troublesome to have interfered with normal daily activity or sleep). Total symptom score (TSS) being the sum of each individual symptom was considered.

Allergen-specific proliferative response

PBMC were isolated by centrifugation on a Ficoll-Hypaque gradient for 30 min at 1800 rpm. PBMC were suspended at 2×10^6 /ml in RPMI 1640 (Sigma, Milan, Italy) supplemented with 10% autologous plasma, 2mm L-glutamine (Sigma), and cultured in duplicate on 96 flat-bottomed plates (Nunc, Kamstrup, Denmark) for 6 days in the presence of allergen extracts (20 mcg/ml), including *Dermatophagoides farinae*, *Parietaria judaica*, five grasses, and cat fur (allergens were kindly provided by Stallergenes Italy, Milan). We chose these allergens as they are the most important cause of sensitization in our area. In all groups, we included duplicate medium controls wells as a negative control and duplicate in presence of 2×10^6 /ml autocleaved bodies of *Candida albicans*, as recall antigen, or PHA. After 6-hrs pulse with 0,5 mCi (3H)-thymidine/well (3H-TdR; Amersham International, Bucks, UK), cultures were harvested and counted incorporated radioactivity as index of proliferation. We expressed data as Kcpm (cpm/1000) in allergen-stimulated wells with medium control subtracted. Data are individual values with median horizontal bars from separate donors of each group

Statistical analysis

We compared proliferation data among groups with the non-parametric Mann-Whitney U test with Prism software (version 3; Graph Pad, San Diego, CA, USA).

RESULTS

Clinical efficacy of SLIT

Allergic subjects undergoing HDM SLIT showed a significant improvement of nasal symptoms in comparison with untreated rhinitics ($p < 0.01$).

Effects of SLIT on the proliferative response

- *Candida albicans*-induced T-cell proliferation (figure 1): SLIT-treated patients showed significant lower proliferation than untreated allergic subjects ($p = 0.0002$). Untreated allergic subjects showed significant higher proliferation than healthy subjects ($p = 0.0006$). There was no difference between SLIT-treated subjects and healthy donors.
- *Dermatophagoides farinae*-induced T-cell proliferation (figure 2): SLIT-treated patients showed lower pro-

liferation than untreated group, even though without significant difference. There was no significant difference between treated rhinitics and normal subjects, whereas there was significant difference between untreated allergics and normal subjects ($p=0.041$).

➤ *Parietaria judaica*-induced T-cell proliferation (figure 2): SLIT-treated rhinitics showed significant lower proliferation than untreated allergic subjects ($p=0.0033$). Untreated allergics showed higher proliferation than control group ($p=0.0033$), whereas there was no difference between normal subjects and SLIT-treated patients.

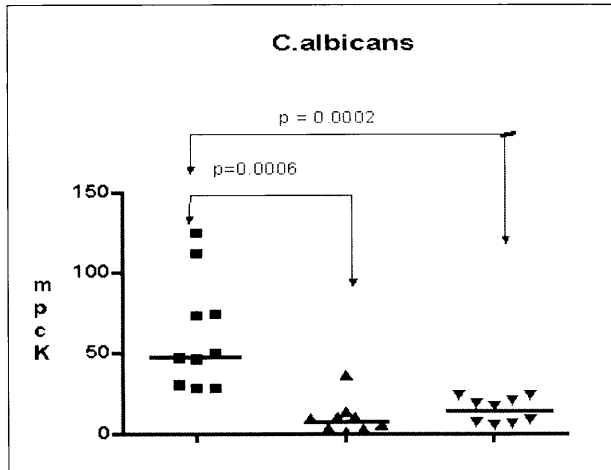


Figure 1: Proliferative response to *Candida albicans* of PBMCs from untreated (UA), normal (CG), and SLIT-treated (TA) subjects. (Kilocounts per minute).

Five grasses-induced T-cell proliferation (figure 2): SLIT-treated patients showed lower proliferation than untreated rhinitics ($p=0.009$). There was no significant difference between treated subjects and healthy volunteers. Untreated allergic patients showed higher proliferation than normal subjects ($p=0.0033$). Cat fur-induced T-cell proliferation (Figure 2): there was no significant difference between treated and untreated patients and between treated subjects and normal controls. Untreated subjects showed higher proliferation than healthy donors ($p=0.033$).

DISCUSSION

Allergic rhinitis is characterized by a Th2-polarized inflammation. Th2-derived cytokines have been previously investigated in subjects with allergic rhinitis, particularly concerning the role of IL-4 and IL-13 (18). IL-4 is a pivotal cytokine that orchestrates allergic inflammation as it is the most important signal to induce Th2 polarization in allergic subjects (19). IL-4 and IL-13 promote IgE synthesis, upregulates adhesion molecules selective for eosinophil recruitment, and cause increased mucus production and airway hyperreactivity (19). Thus, there is agreement about the role exerted both by IL-4 and IL-13 as main pathogenetic factors in inducing, maintaining, and amplifying inflammatory allergic inflammation.

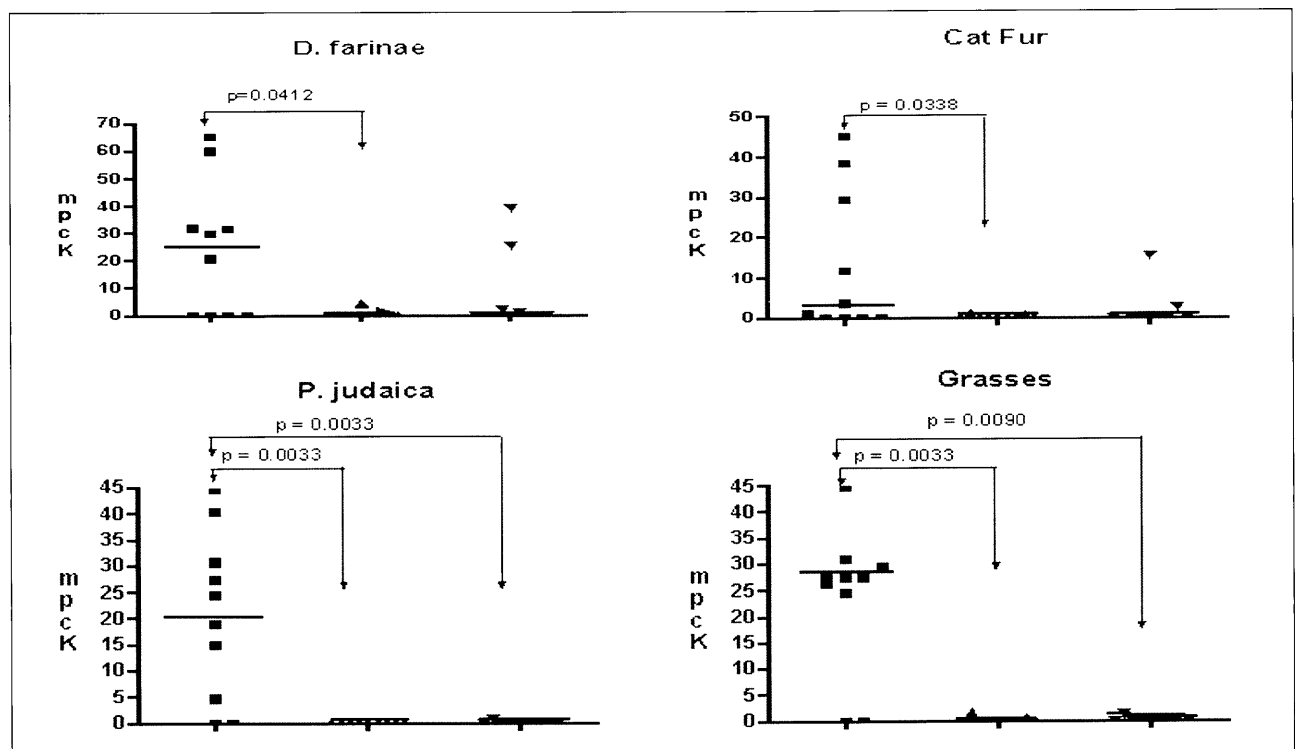


Figure 12: Proliferative response to *Dermatophagoides pteronyssinus*, *Parietaria judaica*, cat fur and grasses of PBMCs from untreated (UA), normal (CG), and SLIT-treated (TA) subjects. (Kilocounts per minute).

Otherwise, there is an accumulating evidence that Th1-related cytokines, such as IFN γ and IL12, may suppress and counteract this Th2 response and viceversa as there is functional dichotomy between Th1 and Th2 cells (20). However, there is evidence that Th1 cells may be themselves pro-inflammatory as it has been demonstrated that the development of an Th1-associated inflammatory response could exacerbate asthma (21). Furthermore, the increase in prevalence of atopic diseases over the past 20 years has been accompanied by an increase in the prevalence of autoimmune, Th1-driven, diseases, also in the same subject (22). Thus, forms of immunity other than pure Th1 responses must be effective in regulating the allergic inflammation: such as regulatory T cells (3). As recently evidenced, allergic subjects shows a defect of T regulatory cells (10). This defect may be replaced by subcutaneous SIT as provided by several studies (6,7,8). Particularly, successfully SIT has been demonstrated capable of inducing IL-10 production by T cells (9). IL-10 exerts a number of documented activities that are relevant for understanding the mechanisms of action of specific immunotherapy. IL-10 properties include: the modulation of allergen-specific IgE production in favor of IgG4 (23), the inhibition of allergen-dependent mast cell activation (24), and the suppression of activation and survival of eosinophils (25). Moreover, IL-10 reduces the production of pro-allergic cytokines (26) and induces hyporeactivity to allergens (27).

Sublingual immunotherapy 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 (11,14). Few studies showed a relevant effect on the IgG4 response after SLIT, thus it could seem that SLIT does not reduce constantly the IgE production and increase the IgG4 synthesis (11). On the contrary, a decrease of serum IL-13 was demonstrated after SLIT therapy (28). Moreover, it has been reported that T cell proliferative response was reduced by SLIT (29). The same effect was demonstrated also for other local routes of specific immunotherapy, such as nasal (16) and oral (30). These findings confirm numerous experimental studies in animal models, showing that the oral route is tolerogenic and can reverse the Th2 polarization (31, 32, 33).

The present study evidenced that allergic subjects successfully treated with SLIT for HDM showed reduced T cell proliferation induced by different stimuli. The most relevant result is the response to *Candida albicans* that is an ubiquitous antigen. We chose this antigen because it is a common recall antigen. It is of note that a previous study did not show reduction of tetanus toxoid-induced proliferation, probably because of low allergen dose schedule (29).

In addition, allergen-specific T cell proliferation was also affected by SLIT. Even though Der f-induced proliferation was not significantly reduced, perhaps because of few cases investigated, it is noteworthy to mention that there was significant reduction of T cell proliferation also for other allergens that were not causal allergens, but only responsible of sensitization. Thus, these findings confirm that SLIT is capable of inducing immunologic hyporeactivity that may be both specific toward the allergen administered and also directed to other environmental antigens. As recently evidenced by several studies (6,7,8), the decreased T cell proliferation induced by SLIT may be consequence of T regulatory cell involvement. Therefore, decreased T cell proliferation may be attributed to the induction of generation of regulatory cells capable of inducing a tolerance to the causal allergen. This tolerance is involved in the clinical improvement achieved by SLIT-treated patients.

In conclusion, we reported systemic immunologic changes induced by the administration of allergenes via the oral mucosa. It is well known that oral-associated lymphoid tissue shows a particular capacity to induce immune tolerance (34,35). According with the studies, analyzing the events occurring during mucosal immunotherapy, such as via nasal (16), oral (17), and sublingual route (29), we conclude that HDM SLIT may induce both a decrease in T cell reactivity and an immunologic tolerance.

References

1. Frew A.-J. - Immunotherapy of allergic disease. *J Allergy Clin Immunol* 2003;111:S712-9.
2. Bousquet J., Lockey R.-F., Malling HJ. WHO position paper. Allergen immunotherapy: therapeutic vaccines for allergic disease. *Allergy* 1998;53(S44):1-42.
3. Akbari O., Stock P., DeKruiff R.-H., Umetsu D.-T. - Role of regulatory T cells in allergy and asthma. *Curr Opin Immunol* 2003;15:627-33.
4. Herrick C.-A., Bottomly K. - To respond or not to respond: T cells in allergic asthma. *Nature Reviews Immunology* 2003;3:1-8.
5. Umetsu D.-T., Akbari O., DeKruiff R.-H. - Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003;112:480-7.
6. Jutel M., Akdis M., Budak F. *et al.* - IL10 and TGF β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003;33:1205-14.
7. Francis J.-N., Till S.-J., Durham S.-R. - Induction of IL10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;111:1255-61.
8. Savolainen J., Laaksonen K., Rantio-Lehtimäki A., Terho E.-O. - Increased expression of allergen-induced in vitro interleukin-10 and interleukin-18 mRNA in peripheral blood mononuclear cells of allergic rhinitis patients after specific immunotherapy. *Clin Exp Allergy* 2004;34:413-9.
9. Blaser K., Akdis C.-A. - Interleukin-10, T regulatory cells and specific allergy treatment. *Clin Exp Allergy* 2004;34:328-31.
10. Ling E.-M., Smith T., Nguyen X.-D. *et al.* - Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;363:608-15.

11. Canonica G.-W., Passalacqua G. - Non-injection routes for immunotherapy. *J Allergy Clin Immunol* 2003;111:437-48.
12. Malling H.-J., Adreth-Vogelers U., Alvarez-Cuesta E., Björkstén B., Bousquet J., Caillaud D. *et al.* - Local immunotherapy (position paper). *J Allergy* 1998;53:933-48.
13. Bousquet J., van Cauwenberg P. - Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001;108:S1-315.
14. Passalacqua G., Lombardi C., Canonica G.-W. - Sublingual immunotherapy: an update. *Curr Opin Allergy Clin Immunol* 2004;4:31-6.
15. Wilson D.-R., Torres L.-I., Durham S.-R. - Sublingual immunotherapy for allergic rhinitis (Cochrane review). *Cochrane Database Syst rev* 2003;(2):CD002893.
16. Giannarini L., Maggi E. - Decrease of allergen-specific-T-cell response induced by local nasal immunotherapy. *Clin Exp Allergy* 1998;28:404-12.
17. TePas E.-C., Hoyte E.-G., McIntire J.-J., Umetsu D.-T. - Clinical efficacy of microencapsulated timothy grass pollen extract in grass-allergic individuals. *Ann Allergy Asthma Immunol* 2004;92:25-31.
18. Bachert C., Van kempen M., Van Cauwenberge P. - Regulation of proinflammatory cytokines in seasonal allergic rhinitis. *Int Arch Allergy Immunol* 1999;118:375-9.
19. Kon O.-M., Kay A.-B. - T cells and chronic asthma. *Int Arch Allergy Immunol* 1999;118:133-135.
20. Tang C., Inman M.-D., van Rooijen N. *et al.* - Th type-1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol* 2001;166:1471-81.
21. Hansen G., Berry G., Dekruiff R.-H., Umetsu D.-T. - Allergen specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 1999;103:175-83.
22. Bach J. - The effects of infections on susceptibility to autoimmune and allergic diseases. *New Engl J Med* 2002;347:911-20.
23. Jeannine P., Lecoanet S., Delneste Y. *et al.* - IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* 1998;160:3555-61.
24. Royer B., Varadaradjalou S., Saas P. *et al.* - Inhibition of IgE-induced activation of human mast cells by IL-10. *Clin Exp Allergy* 2001;31:694-704.
25. Takanasi S., Nonaka R., Xing Z. *et al.* - Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J Exp Med* 1994;180:711-5.
26. Schandene L., Alonso-Vega C., Willems F. *et al.* - B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. *J Immunol* 1994;152:4368-74.
27. Groux H., Bigler M., de Vries J.-E., Roncarolo M.-G. - Interleukin-10 induces a long term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* 1996;184:19-29.
28. Ippoliti F., De Santis W., Volterrani A. *et al.* - Immunomodulation during sublingual therapy in allergic children. *Pediatr Allergy Immunol* 2003;14:216-21.
29. Fanta C., Bohle B., Hirt W. *et al.* - Systemic immunological changes induced by administration of grass pollen allergens via the oral mucosa during sublingual immunotherapy. *Int Arch Allergy Immunol* 1999;120:218-24.
30. TePas E.-C., Hoyte E.-G., McIntire J.-J., Umetsu D.-T. - Clinical efficacy of microencapsulated timothy grass pollen extract in grass-allergic individuals. *Ann Allergy Asthma Immunol* 2004;92:1-2.
31. Mowat A.-M. - The regulation of immune response to dietary proteins. *Immunol Today* 1987;8:93-6.
32. Holt P.-G., Vines J., Britten D. - Sublingual allergen administration. I. Selective suppression of IgE production in rats with high allergen doses. *Clin Allergy* 1988;18:229-34.
33. Chen Y., Kuchroo V.-K., Inobe J. *et al.* - Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 1994;265:1237-40.
34. Husby S., Mestecky J., Moldoveanu Z. *et al.* - Oral tolerance in humans. *J Immunol* 1994;152:4663-70.
35. Weiner L.-W. - Oral tolerance: Immune mechanisms and treatment of autoimmune diseases. *Immunol Today* 1997;18:335-43.

