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Preliminary report

Sublingual immunotherapy reduces soluble HLA-G and HLA-A,-B,-C serum levels in patients with allergic rhinitis

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ABSTRACT

Allergic rhinitis (AR) is characterized by Th2 polarized immune response. Soluble HLA (sHLA) molecules play an immunomodulatory activity. Specific immunotherapy is the only causal treatment for AR. So far no study investigated the effect of sublingual immunotherapy (SLIT) on sHLA molecules.

The aim of the study was to evaluate sHLA-G and sHLA-A,-B,-C serum levels in AR patients with pollen allergy before and after a pre-seasonal course of SLIT. Forty AR patients with pollen allergy were enrolled and they assumed a pre-seasonal SLIT course for 3 months. Serum sHLA-G and sHLA-A,-B,-C and IFN- γ and IL-4 levels were determined by ELISA method at baseline and 3 months after the end of the SLIT course. Symptoms severity was assessed by a Visual Analogue Scale.

Both sHLA-G and sHLA-A,-B,-C levels significantly diminished ($p < 0.0001$ for both) after SLIT. Moreover, there was a highly significant relationship between the serum levels of these two soluble molecules ($r = 0.84$). Significant relationship between symptoms evaluated by VAS and change of sHLA molecules was also evidenced ($r = 0.60$ and 0.63). Serum cytokines were not affected by SLIT.

Therefore, this preliminary study provides the first evidence that both sHLA-G and sHLA-A,-B,-C levels are significantly reduced by SLIT in AR patients with pollen allergy. Therefore, the clinical implication of this study is that these soluble molecules might be interpreted as biomarker of response to SLIT.

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1. Introduction

Allergic rhinitis (AR) is sustained by a mucosal inflammation, orchestrated by T helper (Th) 2 cells [1]. Th2 cells play an important role in the pathogenesis of AR, as IL-4 promotes isotype switching to IgE synthesis, while IL-5 promotes, mostly in the nasal mucosa, eosinophil accumulation and activation [1]. In addition, regulatory T cells (Tregs) play an important role in controlling this Th2-based response, as a matter of fact allergic diseases are presently considered immunoregulatory disorders [2]. In this regard, interest in HLA-G antigens has been showed by the possibility that they may be involved in inflammatory diseases [3]. HLA-G is a human, non classical major histocompatibility complex molecule expressed in human-privileged sites and plays an essential role in the development of maternal tolerance to genetically different foetal tissues [4]. Several HLA-G-mediated mechanisms have been described. Most of them are immunosuppressive, however it has been reported that there is an induction by HLA-G of Th2-like cytokine profile through stimulation of IL-3, IL-4, and IL-10 secretion [5]. Numerous studies provided

evidence that the serum level of the soluble form of HLA-G (sHLA-G) is increased in different pathological conditions, including immune-mediated disorders, transplantations, and malignancies [6]. So far, however, only two studies have been conducted on allergic patients. The first was conducted in children and showed that atopic asthmatics had significantly higher sHLA-G levels than healthy controls [7]. The second provided evidence that patients with allergic rhinitis have higher sHLA-G serum levels than normal subjects [8]. Moreover, it is known that also classical HLA class I molecules, which are characterized by a broad tissue expression and by a high degree of polymorphism, are found in serum of healthy individuals in soluble form (sHLA-A,-B,-C) [9]. The serum level of sHLA-A,-B,-C antigens may increase in physiological and pathological conditions such as pregnancy, acute rejection episodes following organ allografts, autoimmune diseases, viral infections, tumours, and, as very recently evidenced, allergic rhinitis [8,10,11]. Soluble HLA-A,-B,-C molecules may play immunoregulatory activities like inhibition of alloreactive cytotoxic T lymphocytes (CTL) activity, facilitates apoptosis of CD8+ T lymphocytes and NK cells and suppression of allogeneic mixed lymphocyte cultures [12,13].

AR management includes patient education, allergen avoidance, drug therapy, and, when appropriate, allergen-specific immunotherapy [14]. The rationale of sublingual immunotherapy (SLIT) is that oral administration of high-dose allergens may reduce and prevent IgE

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responses achieving clinical tolerance of the causal allergen. SLIT efficacy has been demonstrated in two meta-analyses on patients with AR [15,16]. However, the exact SLIT mechanism of action is not completely understood [17]. Very recently, it has been shown that the appearance of allergen-specific tolerance induction, is evidenced by restored allergen-specific IL-10 and IFN- γ production [18–20]. On the other hand, no study has evaluated the SLIT effects on soluble HLA molecules. Therefore, the aim of the present study was to evaluate whether a pre-seasonal SLIT course may affect sHLA-G and/or sHLA-A,-B,-C serum levels.

2. Methods

2.1. Patients

Forty monosensitized patients (19 males and 21 females) with AR due to pollen allergy were included in the study; 17 of them were allergic only to *Betula alba*, 16 only to *Parietaria judaica*, and 7 only to Graminaceae. Their mean age was 38.86 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 too.

According to validated criteria [21], in order to make a diagnosis of AR, a history of nasal symptoms during pollen season and positive results to skin prick test were considered as sufficient.

As controls, 22 healthy subjects (11 males, mean age 33.8 years) were evaluated at the identical times of year as well.

The study was conducted with the approval of the local Ethics Committee (EC) and performed with the written informed consent of all participants. As the blind method in clinical trials is mandatory only when the primary outcome is a subjective variable [22], this was approved by the EC as an open study for the main reason that the primary outcome was to investigate the effect of SLIT on the sHLA molecules serum levels, such as objective variable measures.

Patients' and controls' blood samples for assessing sHLA-G and sHLA-A,-B,-C and IFN- γ and IL-4 serum levels were performed in standardized manner and collected before initiating SLIT (baseline), and three months after the end of the SLIT pre-seasonal course that lasted 3 months. Sera were separated from all blood samples within 4 h and stored at -30°C until used. Immediately prior to use, serum samples were thawed and centrifuged to remove aggregates.

2.2. Specific immunotherapy

Subjects undergoing SLIT received either a *B. alba* pollen allergen extract (Anallergo, Florence, Italy; 17 cases, cumulative dose was 30 mcg), *P. judaica* (15 cases, cumulative dose was 12 mcg), or a mix of three Graminaceae (7 cases, cumulative dose was 12 mcg) for at least three months before the pollen season, as pre-seasonal immunotherapy. The dose during the maintenance phase was five drops 3 times per week. The ratio between SLIT vs SCIT allergen dose is 3:1.

Allergic symptoms were scored 6 months after SLIT starting. The patient questionnaire included a visual analogue scale (VAS) of nasal symptoms according to validated criteria [23].

2.3. Immunoassays

sHLA-G molecules were determined by a commercially available immunoassay (EXBIO, Praha, Czech Republic) which has been recently validated in a workshop [24]. The assay is based on a technique of DDIA (double determinant immunoassay) using the monoclonal antibody MEM-G/9 (anti HLA-G1/G5) for coating and the monoclonal antibody NAMB-1 (anti- β 2-microglobulin) for detection according to the manufacturer's instructions; results were expressed as ng/mL. sHLA-A,-B,-C molecules were determined by a double determinant immunoassay performed as previously described [25],

with minor modifications, utilizing anti-HLA class I monoclonal antibody W6/32 directed to a framework determinant of all HLA class I molecules and biotinylated anti- β 2-microglobulin monoclonal antibody NAMB-1; results are expressed as ng/mL. The intra-assay (made in triplicates) and inter-assay variation were 3.2 and 13.9% respectively for HLA-G molecules and 5.5 and 15% respectively for total HLA-I molecules.

The human IFN-gamma and IL-4 assays (R&D Systems, Milan, Italy) employ the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for IFN-gamma has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IFN-gamma present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IFN-gamma is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IFN-gamma or IL-4 bound in the initial step. The color development is stopped and the intensity of the color is measured (pg/mL). The minimum detectable dose was typically less than 10 pg/mL.

2.4. Statistical analysis

Descriptive statistics were calculated and reported in terms of frequencies and percentages for qualitative variables and in terms of medians with percentiles.

Comparisons of sHLA-G and sHLA-A,-B,-C levels before and after pre-seasonal SLIT (paired data) were performed by means of the non-parametric Wilcoxon test.

Comparison of quantitative variables among different groups of patients (example: treated patients vs controls) was made by the non

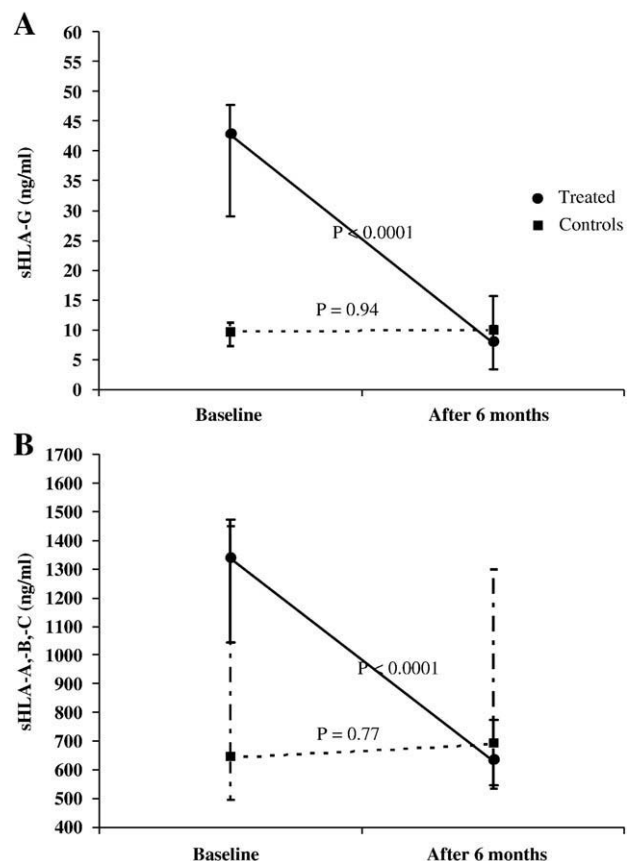


Fig. 1. Serum levels of sHLA-G (panel A) and sHLA-A,-B,-C molecules (panel B) before and after pre-seasonal SLIT in two groups of subjects: treated allergic patients and normal controls. Data are expressed as medians; bars represent first and third quartiles.

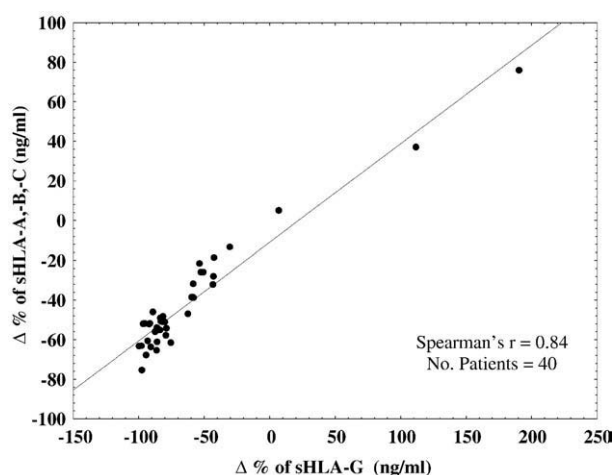


Fig. 2. Correlation between the percentage change (Δ) of sHLA-G and sHLA-A,-B,-C serum levels.

parametric analysis of variance (Kruskal–Wallis test) and the Dunn's test was used as a *post-hoc* test.

The correlation between percentage changes (Δ) of sHLA-G and sHLA-A,-B,-C levels and with symptoms severity and Ig serum levels was made by the non parametric Spearman's correlation coefficient (r_s).

3. Results

The median serum levels of sHLA-G and sHLA-A,-B,-C molecules before and after pre-seasonal SLIT in the treated group of patients and in control group of patients are shown in Fig. 1 (panels A and B, respectively). sHLA-G levels in the treated group of patients were 42.8 ng/mL before and 7.9 ng/mL after SLIT and sHLA-A,-B,-C levels were 1337.5 ng/mL before and 631 ng/mL after SLIT. The decrease of both molecules after SLIT was statistically significant ($p < 0.0001$ for both). On the contrary, sHLA-G levels in the control group of subject remained stable from 9.8 ng/mL to 9.9 ng/mL after SLIT ($p = 0.94$) as well as sHLA-A,-B,-C levels from 642 ng/mL at baseline to 689 ng/mL after SLIT ($p = 0.77$). Baseline sHLA-G levels (42.8 ng/mL) were significantly higher with respect to baseline sHLA-G levels (9.8 ng/mL) in the control group (p value < 0.01). After SLIT sHLA-G levels in the treated group decreased significantly, reaching the sHLA-G levels of the controls; in fact the two levels, after SLIT, were not statistically significant after SLIT. Similar phenomena were observed for and sHLA-A,-B,-C: baseline levels in allergic patients (1337.5 ng/mL) were significantly higher than baseline sHLA-G levels in the control group (642 ng/mL) (p value < 0.05). After SLIT sHLA-A,-B,-C levels in the treated group decreased significantly, reaching sHLA-A,-B,-C levels of the controls (Fig. 1, panel B).

Moreover the percentage changes (Δ) of sHLA-G and sHLA-A,-B,-C serum levels were significantly correlated ($r_s = 0.84$) (Fig. 2).

Furthermore, the percentage changes (Δ) of sHLA-G and sHLA-A,-B,-C serum levels were significantly correlated with a clinical symptoms score measured by a VAS ($r_s = 0.63$ and $r_s = 0.60$, respectively) (Fig. 3).

Median serum IFN- γ levels were not significantly affected by SLIT course: 33.02 pg/mL at baseline and 33.98 after treatment.

Median serum IL-4 levels were not significantly affected by SLIT course: 24.07 pg/mL at baseline and 20.48 after treatment.

4. Discussion

Allergic rhinitis is characterized by a Th2-polarized inflammation. Th2-derived cytokines, such as IL-4 and IL-13, are the main pathogenic

factors able to induce, maintain and amplify the allergic inflammation. IL-4 and IL-13 orchestrate inflammatory reaction by: promoting IgE synthesis, up-regulating adhesion molecules, selective for eosinophil recruitment, and causing increased mucus production and airways hyperreactivity [26]. In addition, a defect of Tregs characterizes allergic disorders, but this defect may be replaced by specific immunotherapy [19]. In this regard, soluble HLA molecules might play an important role in the SLIT-induced mechanisms of immune tolerance toward allergens.

Seven HLA-G isoforms generated by alternative splicing of the primary HLA-G transcript have been described. Four of them, HLA-G1, -G2, -G3, and -G4, are bound to the cell surface, while the remaining three, HLA-G5, -G6, and -G7 are soluble and are the counterparts of HLA-G1, -G2, and -G3 respectively [27,28]. Soluble HLA-G derives from the secretion of soluble isoforms as well as from the shedding of proteolytically cleaved surface isoforms. Like their surface membrane bound counterparts, the major soluble isoforms, i.e. HLA-G1 and HLA-G5, share immunosuppressive activities mediated by their binding to the receptors CD85j, CD85d, CD158d, and CD160. In fact, sHLA-G molecules inhibit T cell proliferation induced by allogeneic dendritic cells [29] and, in analogy to sHLA-A,-B,-C molecules, induce apoptosis of T and NK CD8+ cells and inhibit cytotoxic T cell activity through CD8 ligation [30]. The immunoregulatory characteristics of HLA-G have prompted a number of studies aimed at evaluating the level of sHLA-G in sera from patients affected by a variety of disorders as recently reported in an extensive review [6].

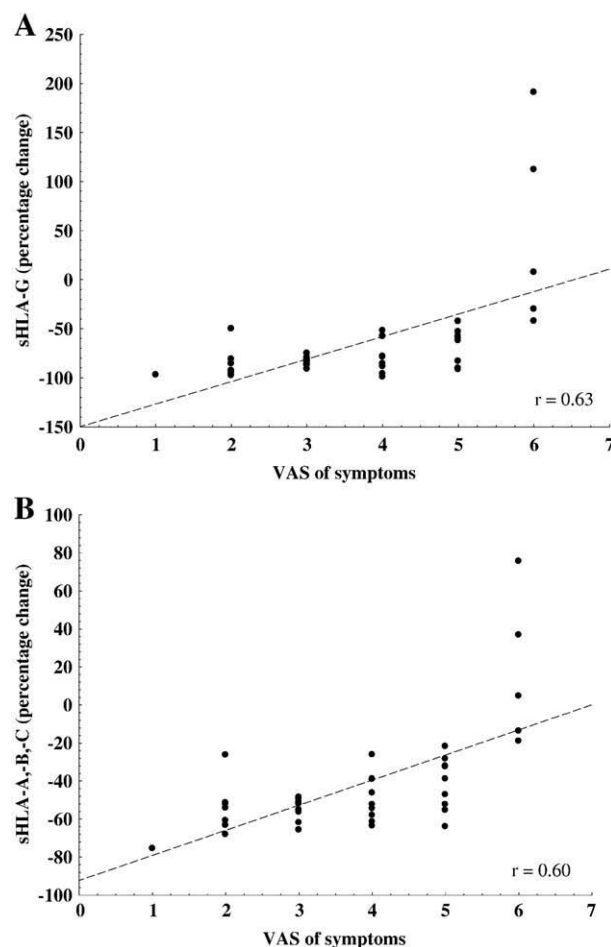


Fig. 3. Correlation between the percentage change (Δ) of sHLA-G (panel A) and sHLA-A,-B,-C (panel B) serum levels and clinical symptoms score measured as a Visual Analogue Scale (VAS).

A previous study reported that atopic children with asthma have higher sHLA-G levels than normal controls [7]. Another study, conducted *in vitro*, showed that there is a defective production of sHLA-G molecules by peripheral blood monocytes in patients with asthma [31]. More recently, it has been evidenced that sHLA-G and sHLA-A, -B, and -C serum levels were significantly higher in patients with allergic rhinitis than in normal subjects [8].

Allergen-specific immunotherapy (SIT) is highly effective in the treatment of AR, and is the only treatment which promotes life-long tolerance against previously disease-causing allergens, through the restoration of normal immunity [32]. SLIT is the preferred version of SIT, with a safer and more effective route of administration, however, its mechanism of action is still poorly understood, as there are few data concerning the immunologic changes induced by SLIT [14]. Regarding the balance between Th1-Th2 cells, some studies have shown a shift from Th2 towards a Th1 response [18,33]. Moreover, a restored Treg function has been reported after SLIT [18,19].

This study was aimed at evaluating sHLA-G and sHLA-A, -B, -C serum levels in a group of patients with AR due to pollen allergy before and after pre-seasonal SLIT. Results show that the serum levels of both soluble HLA molecules significantly decrease after SLIT and that the percentage changes of sHLA-G and sHLA-A, -B, -C molecules are significantly correlated. In addition, normal subjects showed no significant modification of sHLA molecules in identical times of year. On the contrary, a matched group of allergic patients showed a slight serum increase of sHLA molecules during the pollen season, probably due to the exposure to the causal allergen. These findings are in agreement with a published paper reporting that atopic asthmatic children had significantly higher sHLA-G levels than healthy controls [7] and with recent data indicating that sHLA-G and sHLA-A, -B, -C serum levels are increased in the serum of allergic patients in comparison with normal subjects [8]. Moreover, they suggest that soluble HLA molecules may exert an immunoregulatory role in the induction of allergen tolerance induced by SLIT. In *in vitro* experiments it has been shown that elevated HLA-G concentrations induce a Th2-type profile whereas low HLA-G concentrations resulted in a Th1-type profile [34]. Therefore, a reduction of these soluble molecules seems to be related with one of the proposed mechanisms of action of SLIT and in particular with its capacity to shift immune response from Th2 towards a Th1 cytokine secretion profile. Unfortunately, the serum assessment of Th1 and Th2-dependent cytokines failed to demonstrate a SLIT effect. Probably, the possible explanation might be that serum evaluation is scarcely sensible. Indeed, it has been very recently reported that there is a significantly relationship between IFN- γ increased production and reduced levels of soluble HLA molecules after specific immunotherapy [35]. This issue highlights possible functional relationship between soluble HLA molecules and Th1-Th2 balance, confirming previous *in vitro* experiments [34]. Probably, sHLA molecules might induce immune tolerance toward causal allergens allowing a shift from a Th2 polarized response to a Th1 profile, even though functional studies must confirm this hypothesis.

Moreover, the clinical relevance of this study is underlined by the significant relationship between the change of sHLA molecules and the symptoms severity. This issue provides evidence that sHLA molecules might be considered as possible biomarkers of response to SLIT. However, there are some limitations to this study: firstly this is an open study, secondly the control group is numerically limited, and thirdly the duration of specific immunotherapy is short.

In conclusion, the present study has preliminarily evidenced that SLIT is able to decrease sHLA-G and sHLA-A, -B, -C serum levels. Therefore, the clinical implication of this study is that these soluble molecules might be interpreted as biomarker of response to SLIT. However, there is a clear need that these preliminary findings have to be confirmed by further functional studies involving these proteins in relevant *in vitro* systems.

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