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Relationship between soluble HLA-G and HLA-A,-B,-C serum levels, and interferon- γ production after sublingual immunotherapy in patients with allergic rhinitis

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Summary Allergic rhinitis (AR) is characterized by a T-helper (Th)-2 (Th2) polarized immune response. Soluble human leukocyte antigen (sHLA) molecules play an immunomodulatory role. Specific immunotherapy is the only causal treatment for AR and is able to shift the immune response to Th1 polarization. The aim of this study was to evaluate the relationship between sHLA-G and sHLA-A,-B,-C serum levels and interferon- γ (IFN- γ) production in AR patients with pollen allergy before and after a preseasonal course of sublingual immunotherapy (SLIT). A total of 40 AR patients with pollen allergy were enrolled and given a course of preseasonal SLIT for 3 months. Serum sHLA-G and sHLA-A,-B,-C levels were determined by enzyme-linked immunoabsorbent assay, and cell production of IFN- γ was evaluated by enzyme-linked immunoabsorbent spot assay at baseline and 3 months after the end of the SLIT course. There was a significant relationship between sHLA-G serum level change and IFN- γ increase as well as between sHLA-A,-B,-C level change and IFN- γ increase after SLIT. The present study provides the first published evidence that the reduction of sHLA molecules serum levels and the increased IFN- γ production after SLIT in AR patients with pollen allergy are significantly related phenomena.

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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 interleukin (IL)-4 promotes isotype switching to immunoglobulin-E (IgE) synthesis, whereas IL-5 promotes, mostly in the nasal mucosa, eosinophil accumulation, and activation. This inflammatory reaction displays a predominant production of Th2-dependent cytokines over interferon- γ (IFN- γ) expression by Th1, the so-called Th2 polarization [1]. In addition, regula-

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ABBREVIATIONS

AR	allergic rhinitis
HLA	human leukocyte antigen
IFN- γ	interferon- γ
sHLA	soluble human leukocyte antigen
SLIT	sublingual immunotherapy
T2	T-helper-2

tory T cells (T_{regs}) 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, nonclassical, major histocompatibility complex molecule that is expressed in human-privileged sites and that plays an essential role in the development of maternal tolerance to genetically different fetal 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 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 under different pathologic conditions, including immune-mediated disorders, transplantations, and malignancies [6]. So far, however, only two studies have been conducted in allergic patients. The first was conducted in children and showed that atopic asthmatic individuals 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 individuals [8]. Moreover it is known that classical HLA class I molecules, which are characterized by a broad tissue expression and by a high degree of polymorphism, are found in sera of healthy individuals in soluble form (sHLA-A,-B,-C) [9]. The serum level of sHLA-A,-B,-C antigens may increase in physiologic and pathologic conditions such as pregnancy, acute rejection episodes after organ allografts, autoimmune diseases, viral infections, tumors, and, as shown in recent years, allergic rhinitis [10,11]. sHLA-A,-B,-C molecules may play immunoregulatory roles such as inhibition of alloreactive cytotoxic T-lymphocyte (CTL) activity, apoptosis of CD8⁺ T lymphocytes and natural killer cells, and suppression of allogeneic mixed lymphocyte cultures [12,13].

Management of AR 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 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]. 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]. Very recently a preliminary study showed that SLIT is able of reducing sHLA serum levels (manuscript submitted). Therefore the aim of the present

study was to evaluate whether there is a possible relationship between IFN- γ production and sHLA-G and sHLA-A,-B,-C serum levels, respectively, in AR patients after a preseasonal SLIT course.

Subjects and methods

Patients

A total of 40 patients (19 male and 21 female) with AR caused by pollen allergy were included in the study; 17 of them were allergic to *Betula alba*, 16 to *Parietaria judaica*, and seven to *Graminaceae*. Their mean age was 38.86 years (SD, 14.3 years). To be enrolled in the study, patients were required to have AR caused by pollen allergy. Patients using nasal or oral corticosteroids, antileukotrienes and antihistamines within the previous 4 weeks were excluded. Patients with documented allergy to nonpollen allergens were excluded also.

According to validated criteria [21], to make a diagnosis of AR, a history of nasal symptoms during pollen season and positive results upon skin prick test were considered as sufficient. The study was conducted with the approval of the local Ethics Committee and the written informed consent of all participants.

Patient's blood samples for assessing sHLA-G and sHLA-A,-B,-C serum levels and IFN- γ production were collected before initiating SLIT (baseline), and 3 months after the end of the SLIT preseasonal course that lasted 3 months.

Specific immunotherapy

Subjects undergoing SLIT received either a *Betula alba* pollen allergen extract (Anallergo, Florence, Italy; 17 cases, cumulative dose 30 μ g), *Parietaria judaica* (15 cases, cumulative dose 12 μ g), or a mix of three *Graminaceae* (seven cases, cumulative dose 12 μ g) for at least 3 months before the pollen season, as preseasonal immunotherapy. The dose during the maintenance phase was 5 drops three times per week.

Immunoassays

The sHLA-G molecules were determined by a commercially available, recently validated [22], immunoenzymatic assay (EXBIO, Prague, Czech Republic) according to the manufacturer's instructions; results were expressed as nanograms per milliliter. sHLA-A,-B,-C molecules were determined by a double determinant immunoassay, performed as previously described [23] with minor modifications, using anti-HLA class I monoclonal antibody W6/32 and biotinylated anti- β_2 -microglobulin monoclonal antibody NAMB-1. Results are also expressed as nanograms per milliliter.

Enumeration of IFN- γ -specific producing cells by cytokine enzyme-linked immunoabsorbent spot assay

Peripheral blood mononuclear cells (PBMCs) were isolated from a heparinized blood sample by Ficoll-paque (Lymphoflot, Biotest, Germany) density gradient centrifugation. The PBMCs were washed with phosphate-buffered saline and resuspended in complete medium (RPMI-1640 with 2 mmol/l L-glutamine, Invitrogen, Milan, Italy) with 10% autologous plasma at 2×10^6 /ml. Enzyme-linked immunoabsorbent spot assay was performed in accordance with the manufacturer's instructions. Briefly, polyvinylidene difluoride plates (Diacclone, France) were coated overnight with IFN- γ capture mAb. After saturation with RPMI-10% fetal calf serum (Invitrogen), 100 μ l of PBMC suspension was added as well. The cells were stimulated with 1 μ g/ml phytohemagglutinin (Sigma Aldrich, Italy) as

positive control, with allergen extracts used for SLIT for 20 hours at 37°C and 5% CO₂ in humidified air. The allergen concentration of the whole extract of birch, parietaria, and grasses, kindly provided by Anallergo (Florence, Italy) in the final volume of culture medium were respectively 2.5, 1, and 1 µg/ml, based on assessment in preliminary experiments. After incubation, the plates were washed with phosphate-buffered saline-1% bovine serum albumin and incubated with biotinylated detection antibody for 1.5 hours at room temperature. After the washing, streptavidin-alkaline phosphatase conjugate was added for 1 hour at room temperature. Finally, BCIP/NBT was distributed in wells. Enzyme linked immunoabsorbent spot assays were set in duplicates, and detection of spots representing cytokine-producing cells was performed by bioreader (BIO-SYS, Germany).

Statistical analysis

Values at baseline and month 6 were compared using a Wilcoxon test for paired data. The correlation between absolute changes (Δ) in sHLA-G and sHLA-A,-B,-C levels was made by the nonparametric Spearman's correlation coefficient (r).

Results

Soluble-HLA-G and sHLA-A,-B,-C serum levels significantly diminished after SLIT treatment: sHLA-G levels were 35.86 ± 2.7 ng/mL before and 11.36 ± 1.6 ng/mL after SLIT and sHLA-A,-B,-C levels were 1309 ± 73.3 ng/mL before and 695.3 ± 33.2 ng/mL after SLIT. The decrease of both molecules after SLIT was statistically significant ($p < 0.0001$) as shown in Figure 1.

IFN- γ production significantly increased from a mean of 44 ± 68 spots to a mean of 181 ± 89 spots after SLIT ($p = 0.0001$), as shown in Figure 1.

As indicated in Figure 2, there was a significant relationship between sHLA-G change and increase in IFN- γ production after a preseasonal SLIT course ($r = -0.39$, $p = 0.023$).

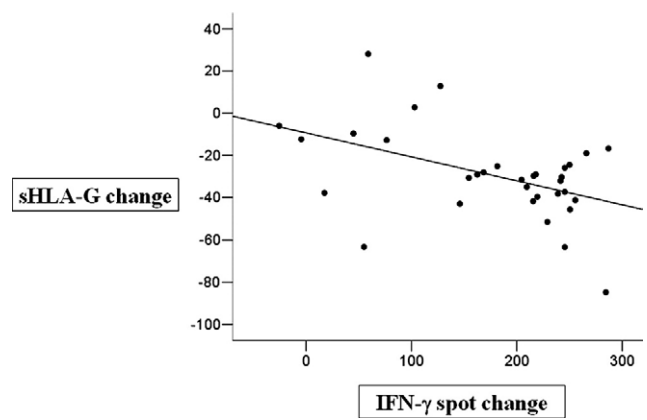


Figure 2. Correlation of difference between sHLA-G serum levels at baseline and month 6 and number of spot changes after SLIT. Negative values represent decreases.

When sHLA-G serum levels tended to decrease, production of IFN- γ tended significantly to increase.

As evident in Figure 3, there was a significant relationship between sHLA-A,-B,-C change and increase of IFN- γ production after a preseasonal SLIT course ($r = -0.38$, $p = 0.029$). When sHLA-A,-B,-C serum levels trend to reduce, the production of IFN- γ significantly trend to increase.

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 this in-

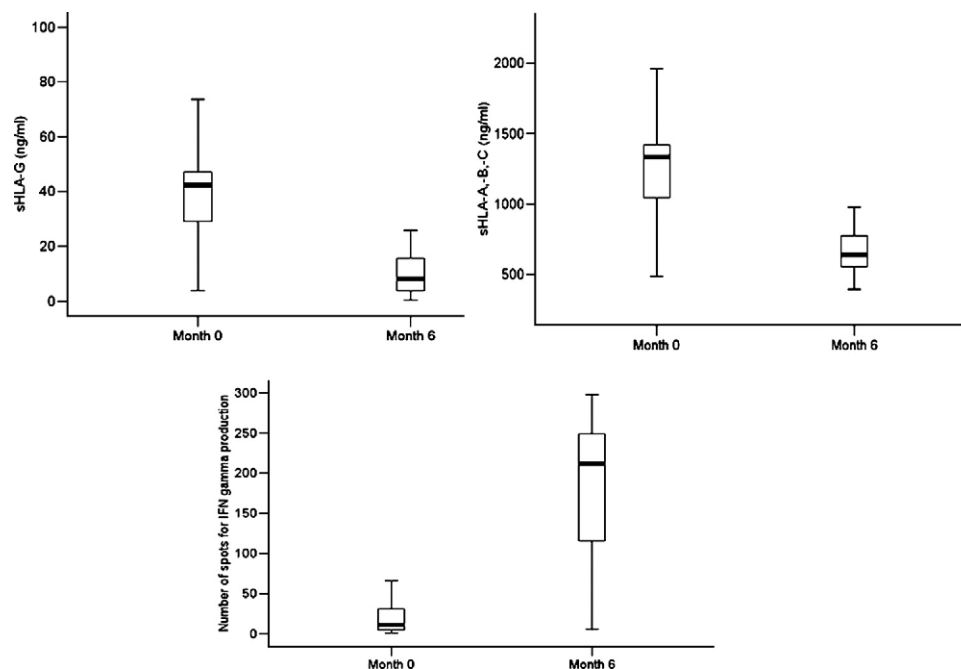


Figure 1. Plots of values of sHLA-G (upper left panel), HLA-A,-B,-C (upper right panel), and IFN- γ (lower panel) at baseline and after 6 months. Plots are based on the median, quartile, and extreme values. Box represents interquartile range, which contains 50% of individual patients' values. Whiskers show highest and lowest values.

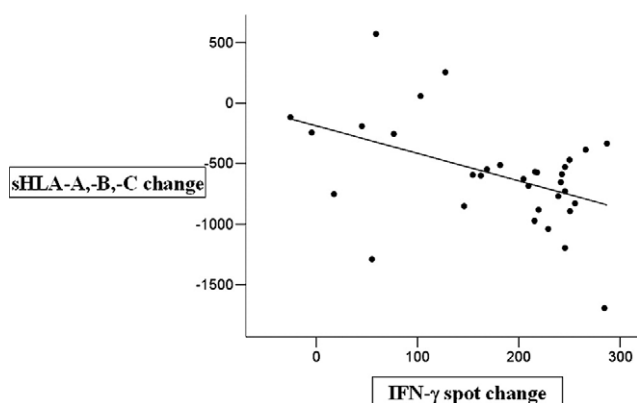


Figure 3. Correlation of the difference between sHLA-A,-B,-C serum levels at month 6 and baseline and number of spot changes after SLIT. Negative values represent decreases.

flammation by promoting IgE synthesis, by up-regulating adhesion molecules selective for eosinophil recruitment, and by causing increased mucus production and hyper-reactivity of the airways [24]. In addition, a defect of T_{reg} characterizes allergic disorders, but this defect may be compensated for 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.

A previous study reported that atopic children with asthma have higher sHLA-G levels than normal controls [7]. Another study, conducted showed that individuals with allergic rhinitis have higher sHLA-G and HLA-A,-B,-C serum levels than 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 [25]. 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 and Th2 cells, some studies have shown a shift from Th2 toward a Th1 response [18,26]. Moreover a restored T_{reg} function has been reported after SLIT [18,19].

This study evaluated the possible relationship between SLIT effects on sHLA-G and sHLA-A,-B,-C serum levels and IFN- γ production in a group of patients with AR caused by pollen allergy, both before and after a preseasonal course of 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 with the increased IFN- γ production. 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 also with very 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 individuals [27].

Moreover, these data suggest that soluble HLA molecules may exert an immunoregulatory effect on 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 [28]. Therefore a reduction of these soluble molecules seems to be related to one of the proposed mechanisms of action of SLIT and, in particular, to its capacity to shift immune response from Th2 toward a Th1 cytokine secretion profile. This phenomenon appears to conflicting with data in the literature supporting a tolerogenic role for soluble HLA molecules. However the model of allergic reaction is different from that in other immunopathologic disorders in that allergic disorders are characterized by a Th2-polarized immune response. This type of immune orientation is also present during pregnancy to protect the fetus from maternal rejection. Indeed, during pregnancy, soluble HLA molecules levels tend to increase [29]. Therefore it seems plausible that improvement in allergic patients after SLIT is characterized by reduction of sHLA molecules and that this phenomenon is related to a typical effect on Th2-Th1 balance such as the increase of IFN- γ production as confirmed in this study.

In conclusion, the present study provides the first published evidence that the reduction of sHLA molecules serum levels and the increased IFN- γ production after SLIT in AR patients with pollen allergy are significantly related phenomena. These preliminary findings need to be confirmed by further studies.

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