

Letter to the Editor

Serum cytotoxic T lymphocyte-associated antigen 4 in hymenoptera venom allergy and its modulation by specific immunotherapy

To the Editor:

Allergy, in a broad sense, encompasses all the clinical manifestations of the “atopic status” that is characterized by an abnormal IgE response to ubiquitous substances, including environmental and food proteins. It is recognized that, when the contact with the offending allergen is occasional and isolated (eg, hymenoptera venom allergy [HVA]), a “pure” IgE-mediated reaction takes place, with a sudden release of mast cell–derived mediators. In these cases specific immunotherapy (SIT), which affects the response to allergens, is highly effective. On the contrary, when the exposure to the allergen is prolonged, a chronic inflammation also occurs. T lymphocytes are responsible for the “deviation” toward the atopic status, and this deviation is essentially driven by regulatory T cells,¹ so that an impairment in the function of regulatory T cells can lead to both autoimmunity and allergy.

Many different signaling molecules intervene in the immune response, and an increasing interest is currently devoted to the activation pathways of T lymphocytes. An effective T-cell activation requires a primary signal delivered by the antigenic peptide presented by major histocompatibility complex molecules and a nonspecific signal (costimulation) mediated by the interaction of CD28 with B7 family members (CD80 and CD86) expressed on antigen-presenting cells.² The cytotoxic T lymphocyte–associated 4 gene (CTLA4), a member of the immunoglobulin supergene family, is able to bind B7 antigens, as is its homolog CD28.² There is evidence that CTLA-4, as distinct from CD28, is a negative regulator of T-cell activation.³ A native soluble form of CTLA-4 (sCTLA-4), lacking the transmembrane sequence, has also been described. High serum concentrations of sCTLA-4 could be detected in patients with various autoimmune disorders, notably autoimmune thyroid diseases,^{4,5} as well as in patients with allergic asthma.⁶ sCTLA-4 might display immunoregulatory functions.^{4,5} The effect of sCTLA-4 binding to CD80/CD86 molecules has been hypothesized to depend on the activation state of the cells involved: thus sCTLA-4 might act indirectly both as an inhibitor or as an enhancer of the immune response.⁶

Based on these background studies, we aimed at investigating the role of CTLA-4 and its possible modulation in a simple model of allergic response: HVA. This was done by measuring sCTLA-4 levels in patients with HVA before and after the induction phase of SIT. Serum IL-10 levels were also assessed as a marker of the immunomodulatory action of SIT, as were specific IgE and IgG4 levels. Nonallergic subjects and patients with respiratory allergy served as control subjects for sCTLA-4 measurement.

Blood samples were collected from adult subjects of both sexes with HVA. They had to have a negative clinical history for asthma, rhinitis, food allergy, or atopic dermatitis and negative skin prick test responses for foods and inhalant allergens. All these patients had a history of systemic reactions to hymenoptera stings and clear sensitization to hymenoptera venom. The diagnosis was made according to European guidelines by using standardized extracts.⁷ Prick tests were performed at increasing concentrations, from 0.01 to 100 µg/mL, and intradermal tests

involved the injection of 0.02 mL of extract at a concentration of 0.001 to 1 µg/mL. The tests were carried out with *Apis mellifera*, *Vespa* species (Stallergènes, Milan, Italy), *Polistes dominulus*, and *Vespa crabro* (Anallergo, Florence, Italy). All these patients were prescribed SIT for the responsible venom. SIT was carried out with a modified rush protocol, involving a build-up phase of 6 weeks and a subsequent maintenance phase with monthly injections of 100 µg of venom. The second blood sample was collected after completing the induction phase of immunotherapy. Healthy subjects had a negative clinical history for rhinitis/asthma, atopic dermatitis, or food allergy and negative skin prick test responses. Patients with respiratory allergy had a positive clinical history for rhinitis, asthma, or both and a positive skin test response for 1 or more of the common inhalant allergens. The standard panel for skin tests included house dust mites, grass, *Parietaria* species, cat and dog dander, olive, birch, hazelnut, *Alternaria* species, egg, milk, codfish, shrimp, and peanut.

sCTLA-4 levels were measured in serum by using an ELISA kit (Bender Med System, Milano, Italy), according to the manufacturer's protocol. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was less than 10% for any reported value. The sensitivity threshold was 0.1 ng/mL. The analytic response was linear, approximately between 0.162 and 1.200 of absorbance values, corresponding to 0.1 to 50 ng/mL (data not shown). Western blotting⁵ was used to confirm the presence of sCTLA-4 in undiluted sera. Proteins were separated by 10% to 20% gradient PAGE in a discontinuous buffer system on a Mini-Protean system (Bio-Rad, Segrate, Milan, Italy), and the components were electroblotted onto polyvinylidene difluoride membranes. The blots were washed, reacted with a 1:100 dilution of the anti-CTLA-4 antibody (clone 14D3, IgG2a; eBiosciences, San Diego, Calif) for 1 hour at room temperature, washed again, and then reacted with reporter antibody (horseradish peroxidase–conjugated anti-rabbit IgG) for 1 hour. The blots were developed by using a commercial chemiluminescence detection kit (BMB, Indianapolis, Ind). Serum IL-10 levels were measured by using an ELISA method (Diacclone Research, Besançon, France). The lower sensitivity threshold of the method was 5 pg/mL. Specific IgE and IgG4 levels were assayed by using ImmunoCAP Phadia, and the IgE/IgG4 ratio was calculated. All results were compared by using the Mann-Whitney test.

Thirty patients (23 male patients; mean age, 45 years; range, 16–65 years) with HVA were included, plus 27 with allergic rhinitis, asthma, or both (12 male patients; mean age, 44 years; range, 21–67 years) and 45 healthy control subjects (34 male patients; mean age, 36 years; range, 19–45 years). The patients with HVA received SIT for the following venoms: 6 for *P dominulus*, 20 for *Vespa* species, 3 for *A mellifera*, and 1 for *V crabro*.

Serum CTLA-4 levels were less than the detection threshold (0.1 ng/mL) of the assay in the majority of healthy subjects (median, 0.1 ng/mL; range, 0.1–36.1 ng/mL) and in patients with respiratory allergy (median, 0.1 ng/mL; range, 0.1–5.8 ng/mL). Only 6 of 45 healthy individuals had detectable sCTLA-4 values, with 1 outlier. On the contrary, sCTLA-4 levels were significantly increased in patients with HVA (median, 17.5 ng/mL; range, 0.1–95.0 ng/mL) and decreased significantly ($P = .004$) at the end of the induction phase of immunotherapy (median, 3.3 ng/mL; range, 0.1–34.1 ng/mL). Accordingly, in those patients at the

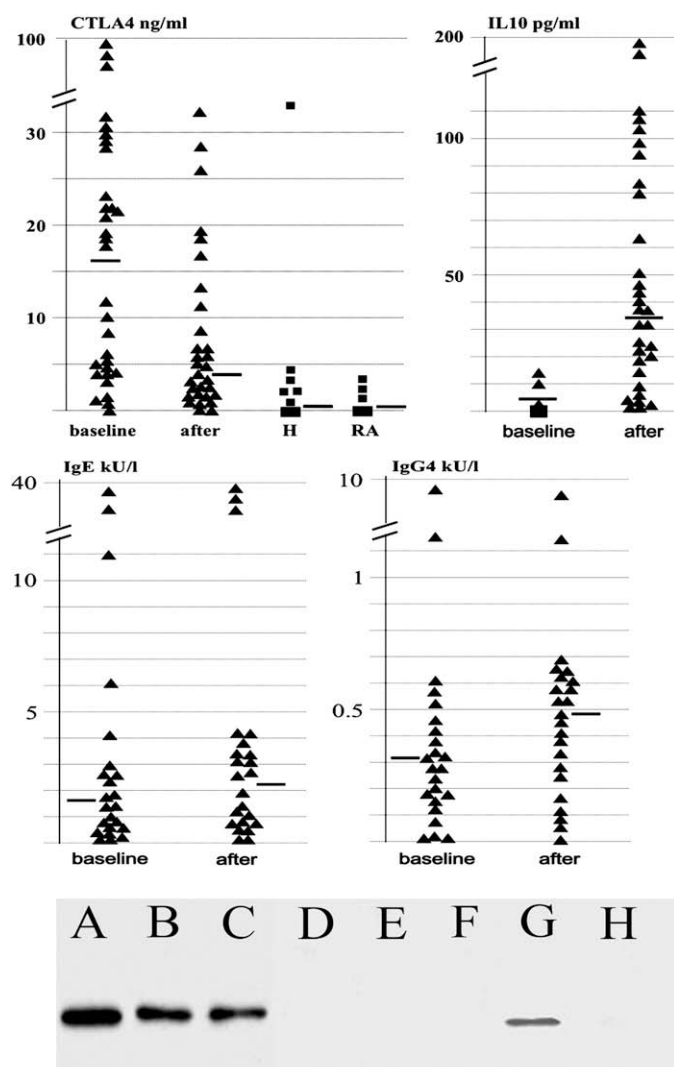


FIG 1. Upper panel: Left, Serum CTLA-4 values before and after immunotherapy induction in patients with HVA, healthy subjects (H), and patients with respiratory allergy (RA). Right, Serum IL-10 values before and after immunotherapy. Middle panel: Specific IgG4 (right) and IgE (left) values before and after immunotherapy. Medians are shown as horizontal thick bars. Lower panel: Western blot of selected sera. Lanes A through C, Patients with HVA (nos. 7, 14, and 22) before SIT; lanes G through I, the same patients after SIT; lanes D and E, patients with respiratory allergy; lane F, healthy subject.

end of the induction phase, the serum levels of IL-10 were significantly increased with respect to baseline values ($P = .0001$). There was no significant change in specific IgG4 levels after SIT (median, 0.48 kU/L; range, 0.02–8.2 kU/L) versus baseline (median, 0.32 kU/L; range, 0.02–9.5 kU/L), and the same happened with specific IgE levels (median, 3.0 vs 1.8 kU/L). The main results are summarized in Fig 1. The specificity of results obtained by means of sCTLA-4 ELISA was confirmed by using Western blot analysis performed on selected samples (Fig 1).

CTLA-4 is recognized as a negative regulator of T-cell activation. In fact, ligation of CTLA-4 to the T-cell surface initiates a cascade of biochemical events that attenuate the ongoing immune response.⁴ The most convincing data that support such a role for CTLA-4 come from experiments in which the *CTLA4* gene is inactivated by the construction of *CTLA4* knockout mice.⁸ These mice display severe polyclonal lymphoproliferative disorders that infiltrate most organs and die in a few

weeks. CTLA-4 might play a role also in atopy, in which the activation of T cells is crucial for the regulation of the immune response. Because CTLA-4 can be present in a native soluble form in the serum and sCTLA-4 is able to bind its physiologic ligands and displays functional activities,^{4,5} we assessed its behavior in a pure IgE-mediated disease: HVA. We showed that sCTLA-4 levels were increased in all patients and significantly decreased after the induction phase of SIT, and their reduction paralleled the increase in IL-10 levels. Therefore as a first consideration, a possible use of sCTLA-4 as a marker of the immunologic effect of SIT can be envisaged. Of note, the change in sCTLA-4 level is an early event that precedes the modifications of specific immunoglobulin. In this regard a possible clinical use might be prospected, especially if any correlation with the clinical responses could be demonstrated in prospective studies. As a second consideration, serum sCTLA-4 concentrations clearly discriminate the patients with HVA with respect to healthy

subjects and patients with allergic respiratory disease. Thus it could be somehow regarded as a disease-specific biomarker: this fact also suggests that HVA, which is a pure IgE-mediated disease, differs in its immunologic regulation from other types of allergic diseases.

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