Basophil expression of diamine oxidase: A novel biomarker of allergen immunotherapy response

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Background: Immunotherapy inhibits basophil histamine release, but the assay is cumbersome, and no one has studied the effects of immunotherapy withdrawal.

Objective: Intracellular fluorochrome-labeled diamine oxidase (DAO) was used as a novel functional readout of basophil histamine release after immunotherapy. Results were compared with conventional basophil surface expression of activation markers.

Methods: Subcutaneous immunotherapy (SCIT)–treated patients (n = 14), sublingual immunotherapy (SLIT)–treated patients (n = 12), participants who completed 3 years of treatment with grass pollen sublingual immunotherapy (the SLIT-TOL group; n = 6), patients with untreated seasonal allergic rhinitis (SAR; n = 24), and nonatopic control subjects (n = 12) were studied. Intracellularly labeled DAO^+ and surface expression of $CD203c^{bright}$, $CD63^+$, and $CD107a^+$ on chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (CRTh2)–positive basophils were measured by means of flow cytometry. Serum IgG_4 levels and serum inhibitory activity for IgE-allergen complex binding to B cells (IgE-FAB) and basophil histamine release were also determined.

Results: Proportions of allergen-stimulated DAO^+CRTh2^+ basophils were higher in participants in the SCIT, SLIT, and SLIT-TOL groups (all $P < .0001$) compared with those in patients in the SAR group. Similarly, there were lower proportions of $CRTh2^+$ basophils expressing surface $CD203c^{bright}$ (all $P < .001$), CD63 (all $P < .001$), and CD107a (all $P < .01$). Rhinitis symptoms were lower in the SCIT, SLIT, and SLIT-TOL groups ($P < .001$) compared with those in the SAR group. Serum inhibitory activity for IgE-FAB and basophil histamine release were also significantly greater in all immunotherapy groups ($P < .05$) compared with the SAR group.

Conclusion: These results support long-term clinical and immunologic tolerance during and after grass pollen immunotherapy. Intracellularly labeled DAO expression by basophils merits further investigation as a surrogate biomarker for monitoring efficacy and tolerance after immunotherapy. (J Allergy Clin Immunol 2015;135:913-21.)

Key words: *Diamine oxidase, basophils, allergen immunotherapy, sublingual immunotherapy, subcutaneous immunotherapy, histamine, basophil activation assay*

In patients with severe hay fever with or without associated seasonal asthma, grass pollen subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) have been shown to be effective and induce long-term clinical and immunologic tolerance.[1-4] Immunologic changes after immunotherapy include suppression of allergen-specific T_H2 responses, induction of regulatory T cells ($IL\text{-}10^+CD4^+CD25^{hi}$, and $CD4^+CD25^{hi}$ forkhead box protein 3–positive cells), and the appearance of "protective" allergen-specific IgG antibodies, particularly of the IgG_4 subclass.[3,5-8] Suppression of the cutaneous early allergic response after immunotherapy is temporally associated with increases in serum IgG-associated inhibitory activity, but the parallel effects of immunotherapy on *ex vivo* allergen-stimulated basophil reactivity have yet to be fully determined.

Basophils were first identified by Paul Ehrlich in 1879 and consist of less than 1% human leukocytes in peripheral blood.[9] They contain cytoplasmic secretory granules, consisting of proteoglycans and histamine.[10] Basophils express FcεRI, which can be cross-linked by allergen-specific IgE after allergen exposure, resulting in degranulation with release of histamine, leukotrienes, and other mediators of the allergic inflammatory response.[11,12] Measurement of histamine release by basophils might be a better functional readout of basophil activation than expression of surface activation markers, such as CD63 (granule-associated tetraspan), CD203c (ectonucleotide pyrophosphatase/phospodiesterase 3, a type II transmembrane ectoenzyme), and CD107a (lysosomal-associated membrane protein 1). However, the current functional immunoassays for measuring histamine release are complex, time-consuming, and poorly reproducible and require an indicator source of whole blood from an atopic subject to test basophil *ex vivo* allergen-induced histamine release. In the mid-1990s, an enzyme-affinity-gold method based on the affinity of diamine

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Abbreviations used

CRTh2: Chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes
DAO: Diamine oxidase
NAC: Nonatopic control subject
PE: Phycoerythrin
RTSS: Rhinoconjunctivitis total symptom score
SAR: Seasonal allergic rhinoconjunctivitis
SCIT: Subcutaneous immunotherapy
SLIT: Sublingual immunotherapy
SLIT-TOL: Participants who completed 3 years of treatment with grass pollen sublingual immunotherapy

oxidase (DAO) for its substrate histamine was used to localize intracellular histamine in mast cells.[12] Subsequently, a DAO-colloidal gold–based technique has also been used to localize histamine within basophils.[13]

We hypothesized that measurement of intracellular fluorochrome-labeled DAO in whole-blood basophils might be a simpler and immediately available functional readout of histamine release at the single-cell level.[14] We postulated that the proportion of DAO^+ chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (CRTh2)–positive basophils would decrease after immediate *ex vivo* allergen stimulation of basophils in patients with grass pollen allergy compared with those in nonatopic control subjects (NACs). We further hypothesized that this decrease would be inhibited in immunotherapy-treated patients. Thus our aim was to both assess the utility of intracellular fluorochrome–labeled DAO compared with surface markers as an indicator of basophil activation in patients with allergic rhinitis and to explore the potential for labeled DAO as a functional biomarker of efficacy and tolerance after allergen immunotherapy. Basophil responsiveness was related to clinical response, serum IgG levels, IgG-associated serum inhibitory activity for IgE-dependent basophil activation, and IgE-allergen complex binding to B cells.

METHODS

Subjects

Patients receiving grass pollen SCIT (*Phleum pratense*; Alutard, ALK-Abelló, Hørsholm, Denmark; n = 14) or grass pollen SLIT (Grazax, ALK-Abelló; n = 12) or participants who completed 3 years of treatment with grass pollen sublingual immunotherapy (the SLIT-TOL group, n = 6), patients with seasonal allergic rhinoconjunctivitis (SAR; n = 24), and NACs (n = 12) provided blood samples and answered symptom questionnaires (Table I). Patients receiving SCIT and SLIT had been receiving immunotherapy for between 12 months and 3 years. Patients in the SLIT-TOL group had completed 3 years of treatment and discontinued immunotherapy 12 to 24 months previously. Blood samples were collected during the grass pollen season (May-July) in 2012 (patients with SAR, n = 12; patients receiving SCIT, n = 7; patients receiving SLIT, n = 6; SLIT-TOL patients, n = 4; and NACs, n = 12) and 2013 (patients with SAR, n = 12; patients with SCIT, n = 7; patients with SLIT, n = 6; and SLIT-TOL patients, n = 2). The study was approved by the South West London REC3 Research Ethics Committee and the Research Office of the Royal Brompton and Harefield NHS Foundation Trust.

Rhinoconjunctivitis symptom scores

Participants were asked to assess the severity of their allergic rhinitis symptoms for the relevant pollen season according to a retrospective symptom questionnaire. This included the 6 categories of runny nose, blocked nose, itchy nose, sneezing, itchy eyes, and watery eyes, each with a rating of 0 (no problem) to 3 (severe problem), resulting in a possible total score of 0 to 18 (rhinoconjunctivitis total symptom score [RTSS]). Participants were specifically asked to evaluate the severity of their symptoms when their allergic rhinitis was at its most severe during the pollen season.

Serum total IgE, specific IgE, and IgG_4 measurements

Total IgE, timothy grass–specific IgE, and specific IgG_4 levels were quantified by using the CAP FEIA system, according to the recommendations of the manufacturer (Phadia, Uppsala, Sweden). Additionally, a multiple-allergen-component analysis was performed for 2 polysensitized patients by using the microarray technique (Immuno Solid Allergen Chip [ISAC]; Thermo Fisher Scientific, Loughborough, United Kingdom), according to the manufacturer's protocol.

Measurements of basophil histamine release by using the DAO flow cytometry assay

Intracellular basophil histamine release after *ex vivo* allergen stimulation was measured by means of flow cytometry with DAO, as kindly provided by Dr Frans Nauwelaers and Dr Noel Drury (BD Biosciences, San Jose, Calif). Phycoerythrin (PE)–conjugated DAO was used to detect histamine release at the single-cell level. $CRTh2^+$ basophils were also immunostained for the surface activation markers CD203c, CD63, and CD107a and acquired on the BD FACSCanto II flow cytometer (BD Biosciences; see the Methods section in this article's Online Repository at www.jacionline.org for further details).

Ex vivo basophil reactivity as measured by CD63, CD203c, and CD107a

Heparinized whole blood (100 μL) was incubated with 0, 0.1, 1, 10, 100, and 1000 ng/mL *P pratense* extract (ALK-Abelló) or anti-IgE (0.5 μg/mL) in a 37°C water bath for 15 minutes. Cells were immunostained with anti-human CD3, CD303, CD294 (CRTh2), CD203c, CD63, and CD107a (all from BD Biosciences). Erythrocytes from whole blood were lysed with BD lysing solution (BD Biosciences) for 10 minutes at room temperature in the dark, samples were centrifuged (for 5 minutes at 200*g*), and supernatants were discarded. The resulting cell pellets were washed in 3 mL of PBS (without Ca^{2+} and Mg^{2+}) and resuspended in 450 μL of ice-cold fixative solution (CellFix, BD Biosciences) before acquisition on the BD FACSCanto II flow cytometer. Nonactivated and activated basophils were identified as $CD203c^{dim}CRTh2^+$ and $CD203c^{bright}CD3^-CD303^-CRTh2^+$ cells, respectively. Additionally, activated cells were also identified as $CD63^+$ and $CD107a^+CD3^-CD303^-CRTh2^+$ basophils. Analyses were performed with BD FACSDiva V6.1.1 software (BD Biosciences).

Inhibition of *ex vivo* basophil reactivity by sera obtained after immunotherapy (SCIT and SLIT)

Heparinized whole blood (100 μL) was incubated with 0, 0.1, 1, 10, 100, and 1000 ng/mL *P pratense* extract (ALK-Abelló) with or without the presence of sera from patients receiving SCIT or those receiving SLIT (100 μL) at 37°C for 15 minutes. Cells were immunostained and acquired by means of flow cytometry, as above.

Basophil histamine release assay

Heparinized whole blood from a subject with grass pollen allergy (specific IgE, >100 kU/L) was incubated with 10 ng/mL *P pratense* extract (ALK-Abelló) in the presence of pre– and post–grass pollen SCIT sera (n = 6). Basophil histamine release into cell-free supernatants was determined by means of ELISA, according to the manufacturer's instructions (IBL, Hamburg, Germany).

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