Marked and persistent eosinophilia in the absence of clinical manifestations

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Background: Although most patients with hypereosinophilic syndromes (HES) present with clinical signs and symptoms attributable to eosinophilic tissue infiltration, some untreated patients remain asymptomatic or have signs and symptoms, such as allergic rhinitis, for which the relationship to peripheral eosinophilia is unclear (hypereosinophilia of unknown significance [HE_{US}]).
Objective: To identify and characterize subjects with HE_{US} of 5 years duration or more as compared to untreated patients with symptomatic HES and healthy normal volunteers.
Methods: All subjects with eosinophilia underwent yearly evaluation, including a standardized clinical evaluation, whole blood flow cytometry to assess lymphocyte subsets and eosinophil activation, and serum collection. Peripheral blood mononuclear cells were cultured overnight with and without phorbol 12-myristate 13-acetate/ionomycin. Cytokines and chemokines were measured in serum and cell supernatants, and mRNA expression was assessed by using quantitative real-time PCR.
Results: Eight of the 210 subjects referred for the evaluation of eosinophilia (absolute eosinophil count [AEC] > 1500/μL) met the criteria for HE_{US} of 5 years duration or more (range, 7-29 years). Peak eosinophil count and surface expression of eosinophil activation markers were similar in subjects with HE_{US} and in untreated subjects with platelet-derived growth factor alpha–negative HES (n = 28). Aberrant or clonal T-cell populations were identified in 50% of the subjects with HE_{US} as compared to 29% of the subjects with HES ($P = .12$). Increased levels of IL-5, GM-CSF, IL-9, and IL-17A were also comparable in subjects with HE_{US} and HES. Serum levels of IgE and IL-13 were significantly increased only in subjects with HES.
Conclusions: A small number of patients with persistent peripheral eosinophilia (AEC > 1500/μL) appear to have clinically benign disease. (J Allergy Clin Immunol 2014;133:1195-202.)

Key words: *Eosinophil, hypereosinophilic syndrome, cytokine, pathogenesis*

Hypereosinophilic syndromes (HES) are a heterogeneous group of rare disorders characterized by marked eosinophilia and a wide array of clinical manifestations. In recent years, there has been considerable debate regarding the definition and classification of HES, due in large part to the identification of specific etiologies for subsets of patients presenting with characteristic signs and symptoms of HES and the availability of targeted therapies that have the potential to prevent morbidity and mortality when instituted early. As a result, a number of new definitions and classifications have been proposed.[1-3]

Although most of these newer classifications allude to a group of patients with marked eosinophilia (absolute eosinophil count [AEC] > 1500/μL) in the absence of clinical manifestations (hypereosinophilia of unknown significance or HE_{US}), little is known about the characteristics and long-term prognosis of such patients in the absence of therapy. More importantly, factors predictive of disease progression have not been identified. In the present study, we describe a cohort of patients with persistent marked eosinophilia who have remained asymptomatic and without end-organ manifestations of eosinophilic disease for more than 5 years in the absence of therapy. Clinical and immunologic features of these subjects are compared with those of untreated patients with HES and normal controls.

METHODS

Study subjects

Two hundred and ten subjects aged 14 years or older with unexplained peripheral eosinophilia (AEC > 1500/μL) underwent detailed clinical and laboratory evaluation between January 1991 and December 2011 under an institutional review board–approved protocol designed to evaluate subjects with eosinophilia (NCT00001406). Subjects on treatment at the time of evaluation (n = 159) or found to be positive for the Fip1-like1 (*FIP1L1*)/platelet-derived growth factor alpha (*PDGFRA*) fusion (n = 15) were excluded from the study. Healthy volunteers without eosinophilia were recruited under an institutional review board–approved protocol to obtain normal blood samples for *in vitro* research (NCT00090662). All subjects gave written informed consent.

Clinical and laboratory assessments

All subjects with eosinophilia underwent yearly evaluation that included complete history and physical examination, routine laboratory testing, serum tryptase, B12, and quantitative immunoglobulin levels, electrocardiogram,

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Abbreviations used
AEC: Absolute eosinophil count
FIP1L1: Fip1-like1
GM: Geometric mean
HES: Hypereosinophilic syndromes
HE_{US}: Hypereosinophilia of unknown significance
PDGFRA: Platelet-derived growth factor alpha
PMA: Phorbol 12-myristate 13-acetate
TARC: Thymus and activation regulated chemokine

echocardiogram, and pulmonary function tests. T-cell receptor-γ gene rearrangement studies were performed yearly as previously described.[4] This method can detect a clonal population representing 2% to 5% of total T cells and identifies approximately 95% of all T-cell receptor-γ rearrangements that occur in clonal T-cell proliferation. The presence of aberrant T cells was also assessed yearly by whole blood flow cytometry (for detailed methodology, see Online Repository at www.jacionline.org). All subjects underwent bone marrow biopsy (or review of prior bone marrow biopsy), testing for *FIP1L1/PDGFRA*, and chest/abdomen/pelvis computed tomography scan at baseline to exclude occult malignancy. Additional testing to assess end-organ involvement was performed as clinically indicated.

Surface expression of eosinophil activation markers

Surface expression of HLA-DR, CD25, and CD69 on peripheral blood eosinophils was assessed by whole blood flow cytometry, as described previously[5] (for detailed methodology, see Online Repository). The normal ranges for surface receptor expression represent the 95% CIs for percent expression on eosinophils from blood bank normal volunteers.

Culture of PBMCs for the generation of supernatants and RNA

PBMCs were isolated by density gradient separation (Ficoll-Paque PLUS; GE Healthcare, Uppsala, Sweden). Red blood cells were lysed with ACK Lysing Buffer (Quality Biological, Inc, Gaithersburg, Md). Cells were washed with 1× PBS and cryopreserved in liquid nitrogen. After thawing, cells were cultured at 2×10^6 cells/mL in 24-well plates in RPMI 1640 supplemented with 10% FCS (Biowhittaker, Walkersville, Md), 80 μg/mL gentamicin (Cellgro, Manassas, Va), 10 mM HEPES (Quality Biological, Inc, Gaithersburg, Md), 1 mM Na-pyruvate (Cellgro), 2 mM L-glutamine (Invitrogen, Carlsbad, Calif) overnight at 37°C, 5% CO_2. PBMCs were subsequently cultured for 6 hours in the presence or absence of 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin (Sigma-Aldrich, St Louis, Mo). Supernatants were collected and frozen at −80°C for future analysis.

Analysis of cytokine and chemokine levels in serum and supernatants

Cytokine and chemokine levels were measured in serum and supernatants by using suspension array technology in multiplex using a Milliplex kit for human IL-2 (supernatants only), IL-5, IL-6 (supernatants only), IL-8, IL-9, IL-10, IL-13, IL-17A, IFN-γ, TNF-α, GM-CSF, and eotaxin (Millipore Corp, St Charles, Mo) according to the manufacturer's instructions. Minimal detectable levels were as follows: IL-2 (0.4 pg/mL), IL-5 (0.1 pg/mL), IL-6 (0.4 pg/mL), IL-8 (0.3 pg/mL), IL-9 (1.1 pg/mL), IL-10 (0.3 pg/mL), IL-13 (0.3 pg/mL), IL-17A (0.4 pg/mL), IFN-γ (0.4 pg/mL), TNF-α (0.2 pg/mL), GM-CSF (2.3 pg/mL), and eotaxin (2.1 pg/mL). For culture supernatants, results are expressed as net levels of production (PMA/ionomycin stimulated – unstimulated). Thymus and activation regulated chemokine (TARC; CCL17) concentration was measured in serum in duplicate using the DuoSet CCL17 ELISA kit (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. The minimum detectable concentration was 7.8 pg/mL.

Quantitative RT-PCR

RNA was isolated using Trizol Reagent. cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif) according to the manufacturer's protocol. Quantitative real-time PCR was performed on an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems) in a reaction volume of 10 μL containing 1× Taqman Fast Universal PCR Master Mix (Applied Biosystems), 1× primer and probe sets Hs00174122_m1 (IL-4), Hs00174200_m1 (IL-5), Hs00174103_m1 (IL-8), Hs00914237_m1 (IL-9), Hs00961622_m1 (IL-10), 4327046F (IL-13), Hs00174383_m1 (IL-17A), Hs00989291_m1 (IFN-γ), Hs00174128_m1 (TNF-α), or 4319413E (18S rRNA) (Taqman Gene Expression Assays; Applied Biosystems), and 50 ng of cDNA. All amplification reactions were performed in triplicate, and the relative quantification of gene expression was normalized to the endogenous control 18S rRNA and expressed as 1/Δ cycle threshold. Undetermined cycle values were given a cycle threshold of 40. Data processing was performed using ABI PRISM SDS software, version 2.3 (Applied Biosystems).

Statistical analysis

Statistical analyses were performed using the nonparametric Mann-Whitney *U* test for comparisons of group means and Fisher exact test for comparison of proportions. Paired samples were compared by using Wilcoxon signed-rank test. A *P* value of less than .05 was considered statistically significant for all analyses.

RESULTS

Demographic and clinical characterization of study subjects

Among the 36 *FIP1L1/PDGFRA*-negative subjects with unexplained eosinophilia (AEC > 1500/μL) who were on no treatment at the time of initial evaluation, 8 (22%) were asymptomatic (HE_{US}) and remained without clinical or laboratory evidence of end-organ manifestations for a minimum of 5 years (median, 11 years; range, 7-29 years). In all 8 subjects, eosinophilia was first identified on a routine complete blood cell count and prompted referral for additional evaluation. The remaining 28 subjects had clinical manifestations of eosinophilic disease (see Table E1 in this article's Online Repository at www.jacionline.org) and were referred for evaluation of HES.

Demographic and laboratory characteristics of the study subjects are shown in Table I. Similar to the subjects with HES, subjects with HE_{US} were predominantly male. Eosinophilia was first identified at a median age of 37 years (range, 16-52 years) in subjects with HE_{US} as compared to 40 years (range, 11-82 years) in subjects with HES ($P = .53$), and the AEC recorded during participation in the research protocol was comparable in subjects with HE_{US} and HES (geometric mean [GM] peak AEC of 3961/μL vs 5122/μL, respectively; $P = .56$). Four subjects (50%) with HE_{US} (subjects 1, 2, 7, and 8) and 8 subjects (29%) with *PDGFRA*-negative HES had laboratory features consistent with a diagnosis of lymphocytic variant HES,[6] including a clonal T-cell receptor rearrangement pattern detected by PCR and/or an aberrant $CD3^-CD4^+$ T-cell population identified by flow cytometry ($P = .40$). GM IgE levels were significantly higher at presentation in subjects with HES than in those with HE_{US} (625 vs 98 IU/mL, respectively, $P = .045$; Fig 1, *A*). Elevated serum IgE levels (>150 IU/mL) were also more common in subjects with HES (24 of 28 as compared to 4 of 8 subjects with HE_{US}, $P = .05$). No subject with HE_{US} and only 2 subjects with *FIP1L1/PDGFRA*-negative HES had serum B12 levels of more than 2000 pg/mL.