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 2013;■■■:■■■-■■■.)

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.

TABLE I. Demographic and clinical characteristics of the study subjects

	HE_{US} (n = 8)	HES (n = 28)	Normal (n = 27)
Median age at initial presentation* (range)	37 (16-52)	40 (11-82)	NA
Gender (male/female)	6/2	19/9	23/4
GM peak AEC/μL (range)	3961† (1,856-7,170)	5,122† (1,530-45,990)	131 (46-312)
Clonal T-cell population	4 (50%)	6 (22%)	ND
Aberrant T-cell phenotype	3 ($CD3^-CD4^+$)	3 ($CD3^-CD4^+$)	ND
GM serum IgE (U/mL) (range)	98‡ (18-864)	625 (22-50,916)	ND
GM serum TARC (pg/mL) (range)	1281 (102-12,005)	1,841† (100-124,270)	248 (19-1,199)
Serum TARC > 1000 pg/mL (%)	5/8 (62%)	14/24† (58%)	2/20 (10%)
Serum B12 >2000 pg/mL	0/8	2/26	ND

NA, Not applicable; *ND*, not done.
*First documented AEC > 1500/μL.
†$P < .05$ as compared to normal controls.
‡$P = .04$ compared to HES.

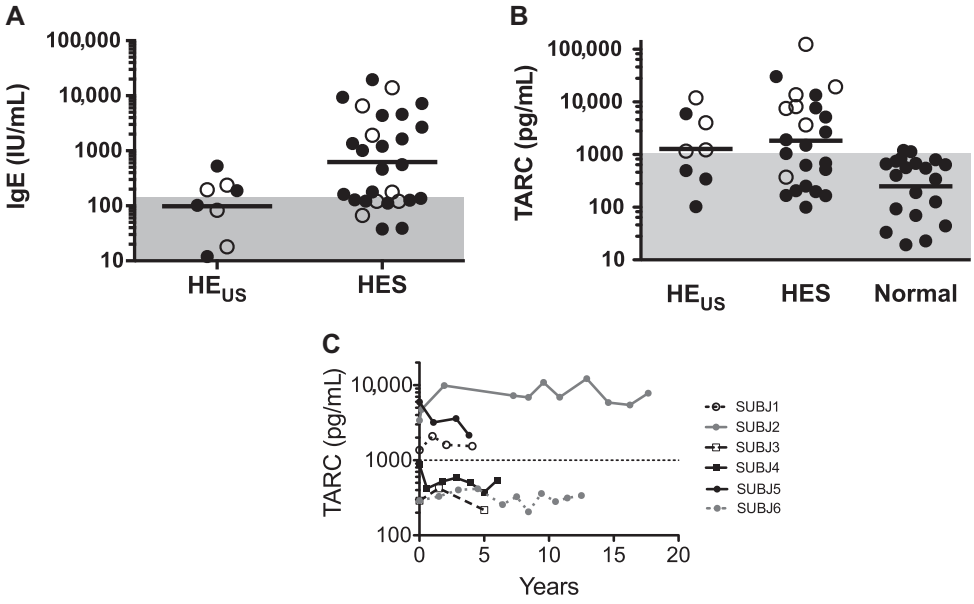

FIG 1. Serum IgE and TARC levels. **A,** Serum IgE levels in subjects with HE_{US} (n = 8) and untreated HES (n = 28). **B,** Serum TARC levels in subjects with HE_{US} (n = 8) and untreated HES (n = 24) and in normal controls (n = 20). *Open circles* are used to denote subjects with clonal and/or aberrant T-cell populations. GMs are indicated by *horizontal bars*. Serum IgE level of less than 150 IU/mL (Fig 1, *A*) and serum TARC level of 1000 pg/mL or less (Fig 1, *B*) are indicated by *gray shading*. **C,** Serum TARC levels over time in 6 subjects with HE_{US}. A TARC level of 1000 pg/mL is indicated by the *horizontal dashed line*. *SUBJ,* Subject.

Eosinophilia and eosinophil activation in subjects with HE_{US}

The AEC decreased over time in 6 of the 8 subjects with HE_{US}, reaching normal levels 8 years after presentation in subject 3 in the absence of glucocorticoids or other therapies with the potential to decrease peripheral eosinophilia (Fig 2). Although a similar pattern was seen initially in subject 2, with a normal AEC documented 14 years after presentation, the decrease in AEC occurred in the setting of an intra-articular steroid injection for hip pain. His counts subsequently rebounded and have remained above 2000/μL since that time.

Eosinophil activation, as assessed by surface expression of CD25, CD69, and/or HLA-DR on unstimulated eosinophils in whole blood, was measured at presentation in subjects with HE_{US} and HES (Fig 3). The number of subjects with increased expression of CD25, CD69, and HLA-DR was comparable between the 2 groups (5 of 8 vs 18 of 26 for CD25, 4 of 8 vs 18 of 26 for CD69, and 3 of 8 vs 9 of 25 for HLA-DR; $P = 1.0$, .4, and 1.0, Fisher exact test). GM expression levels of all 3 activation markers were also comparable between the 2 groups. The presence of a clonal T-cell rearrangement and/or $CD3^-CD4^+$ aberrant T-cell population, as indicated by the open circles, did not appear to correlate with eosinophil surface marker expression in either group. Longitudinal assessment of the same markers revealed intermittent increased expression of CD25, CD69, and HLA-DR (indicated by the black bars in Fig 2) on eosinophils from all 8 subjects with HE_{US} that appeared to correlate in some, but not all instances, with AEC.

Elevated serum TARC levels are associated with T-cell clonality in HE_{US}

Because elevated serum TARC levels have been associated with lymphocytic variant HES[4,7] and 4 of 8 subjects with HE_{US}

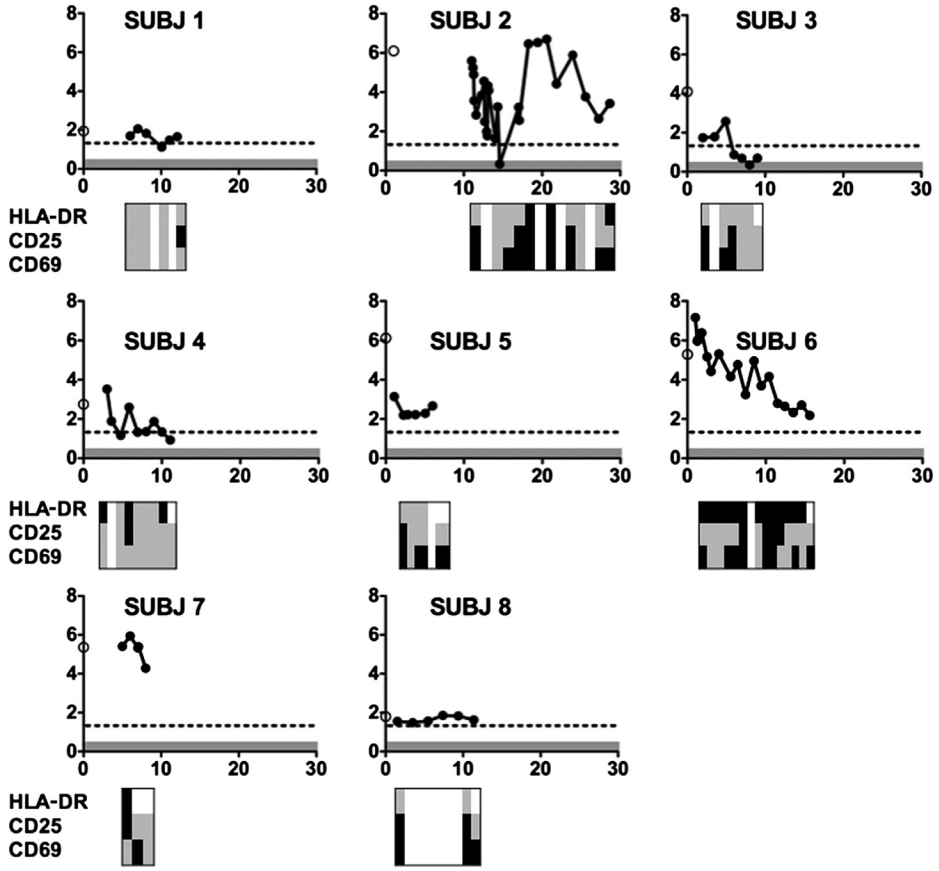

FIG 2. AEC and eosinophil activation over time. *Open circles* indicate the first documented AEC for an individual subject. The *gray shading* represents a normal AEC (<400/mm^3), and the *dashed lines* represent the HES-defining AEC of 1500/μL. Eosinophil expression of HLA-DR, CD25, and CD69 at each time point is depicted in the *boxes* beneath each graph. Above-normal expression is indicated in *black*, normal in *gray*, and no data collected in *white*. *SUBJ*, Subject.

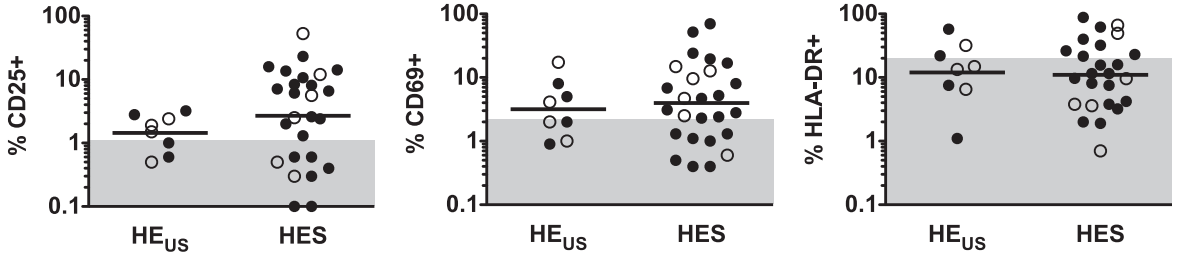

FIG 3. Eosinophil surface activation marker expression. The percentage of eosinophils expressing CD25, CD69, or HLA-DR is shown for subjects with HE$_{US}$ (n = 8) or untreated HES (n = 26). *Open circles* are used to denote subjects with clonal and/or aberrant T-cell populations. GMs are indicated by *horizontal bars*, and the normal range is indicated by *gray shading*. *P* = not significant for all comparisons.

were found to have clonal T-cell receptor rearrangements and/or aberrant $CD3^-CD4^+$ lymphocyte populations consistent with this diagnosis, serum TARC levels were assessed in subjects with HE$_{US}$ and HES and in normal controls (Table I; Fig 1, *B*). At the time of presentation to the National Institutes of Health, serum TARC levels were more than 1000 pg/mL in 5 of 8 (62%) and 14 of 24 (58%) subjects with HE$_{US}$ and HES, respectively, including all but one of the subjects with a documented clonal and/or aberrant T-cell population. Only 2 of 20 (10%) normal subjects had serum TARC levels that exceeded 1000 pg/mL (1199 and 1131 pg/mL). GM TARC levels at the time of presentation were increased in subjects with HES (1841 pg/mL) and HE$_{US}$ (1281 pg/mL) as compared to normal subjects (249 pg/mL), although this difference was statistically significant only for subjects with HES ($P = .001$). Among the subjects with eosinophilia (HES or HE$_{US}$), the presence of an aberrant or clonal T-cell population was associated with increased GM serum TARC levels (6627 pg/mL vs 901 pg/mL in subjects with eosinophilia without an aberrant or clonal T-cell population, $P = .005$). Serum TARC levels were measured yearly in 6 of the 8 subjects with HE$_{US}$ (Fig 1, *C*) but were not correlated with AEC in individual subjects and remained remarkably constant over time.

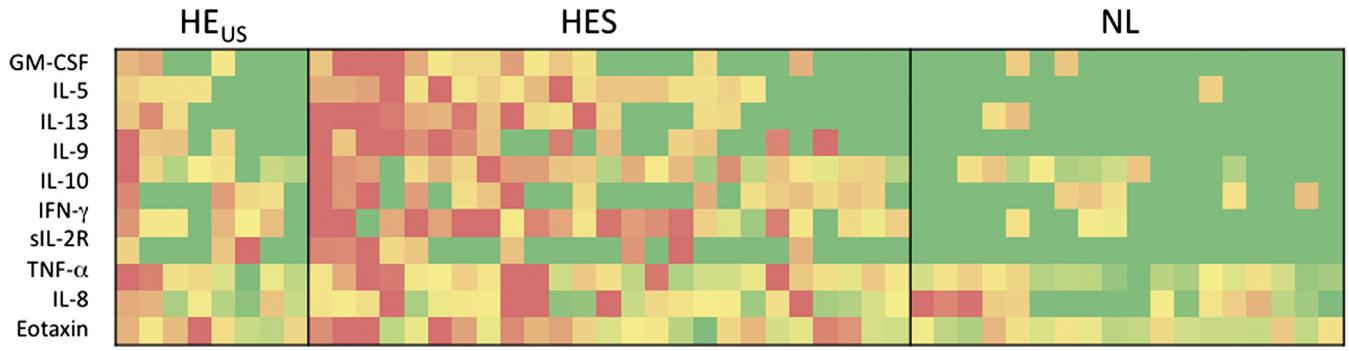

FIG 4. Serum cytokine and chemokine levels. Each *box* represents the value for a given cytokine for an individual subject with HE$_{US}$, untreated HES, and normal controls (*NL*). The color of the box reflects the serum level for the individual subject compared with levels of the same cytokine in all the other subjects, ranging from *dark green* (no measurable analyte) to *dark red*.

TABLE II. GM serum cytokine and chemokine values in subjects with HE$_{US}$ and HES and in normal controls

Serum analyte (pg/mL)	HE$_{US}$ (n = 8)	*P* value*	HES (n = 25)	*P* value*	Normal (n = 18)
GM-CSF	3.6	NS	5.6	.004	2.6
IL-5	0.8	.01	4.4	<.0001	0.1
IL-9	2.8	.002	4.1	<.001	1.1
IL-10	6.5	NS	14.7	<.0001	1.5
IL-13	1.1	NS	3.3	.001	0.4
IL-17	1.2	.008	1.4	.005	0.4
IFN-γ	2.1	NS	3.5	.02	0.9
sIL-2 receptor	58.6	.002	160.1	<.0001	10.3
TNF-α	14.2	NS	25.2	<.001	8.4
IL-8	56.5	NS	75.1	NS	15.3
Eotaxin	122.2	NS	122.1	.02	83.3

NS, Not significant.
*Compared to normal controls, uncorrected for multiple comparisons, NS is defined as >.05.

Serum cytokine profile

Serum cytokine and chemokine levels were measured in subjects with HE$_{US}$ (n = 8) and HES (n = 25) and in normal controls (n = 18). Although serum cytokine levels showed considerable variability among individual subjects with eosinophilia, detectable levels of serum IL-5, IL-9, and IL-17A were seen almost exclusively in subjects with eosinophilia (1 healthy control had a serum IL-5 level of 12 pg/mL) (Fig 4). GM levels of these 3 cytokines, as well as GM levels of soluble IL-2 receptor, were significantly increased in subjects with HE$_{US}$ and HES as compared to healthy controls (Table II). GM levels of serum GM-CSF, IL-10, IL-13, IFN-γ, TNF-α and eotaxin were also significantly increased in subjects with HES as compared to control subjects. No significant differences were detected between subjects with HE$_{US}$ and HES in the GM serum levels of any of the cytokines or chemokines tested. Furthermore, none of the serum cytokine levels was correlated with AEC in subjects with HE$_{US}$ and HES. Serum IL-13 levels were correlated with IgE levels in subjects with eosinophilia (Spearman $r = 0.39$, $P = .02$).

Cytokine and chemokine production by PBMCs

Because eosinophils themselves can be sources of a wide variety of cytokines, PBMCs from subjects with HE$_{US}$ (n = 6) and HES (n = 6) or normal controls (n = 25) were stimulated with PMA/ionomycin and/or staphylococcal enterotoxin B to assess the potential contribution of PBMCs to the observed elevations in serum cytokine levels. GM net (stimulated − unstimulated) levels of GM-CSF, IL-9, and IL-17A were increased in supernatants from PBMCs in subjects with HE$_{US}$ and HES, as compared to normal controls (Fig 5, *A*). GM net levels of IFN-γ and IL-5 were significantly increased only in PBMC supernatants from subjects with HES as compared to controls (Fig 5, *A*). Although the GM net IL-8 level in PBMC supernatants was increased in the normal control group (15.1 pg/mL) as compared to that in subjects with HES (7.9 pg/mL), IL-8 levels were significantly elevated in supernatants from unstimulated PBMCs from subjects with eosinophilia (GM 17.9 ng/mL in HE$_{US}$ and 13.6 ng/mL in HES) as compared to normal controls (4.8 ng/mL; $P < .01$ and $P = .04$, respectively; Fig 5, *B*). GM net levels of IL-2, IL-13, TNF-α, and IL-10 were comparable between the groups.

Expression of cytokine and chemokine mRNA was assessed by quantitative real-time PCR in unstimulated and PMA/ionomycin-stimulated PBMCs after 6 hours of culture. GM net IL-17A mRNA expression was increased 4-fold in PBMCs from subjects with HES as compared to normal controls ($P = .006$). No differences were detected in GM net PBMC mRNA expression of IL-4, IL-5, IL-8, IL-9, IL-10, IL-13, IFN-γ or TNF-α between any of the 3 subject groups.

DISCUSSION

A subset of patients with asymptomatic eosinophilia (AEC > 1500/μL) (HE$_{US}$) have been alluded to in a number of recent

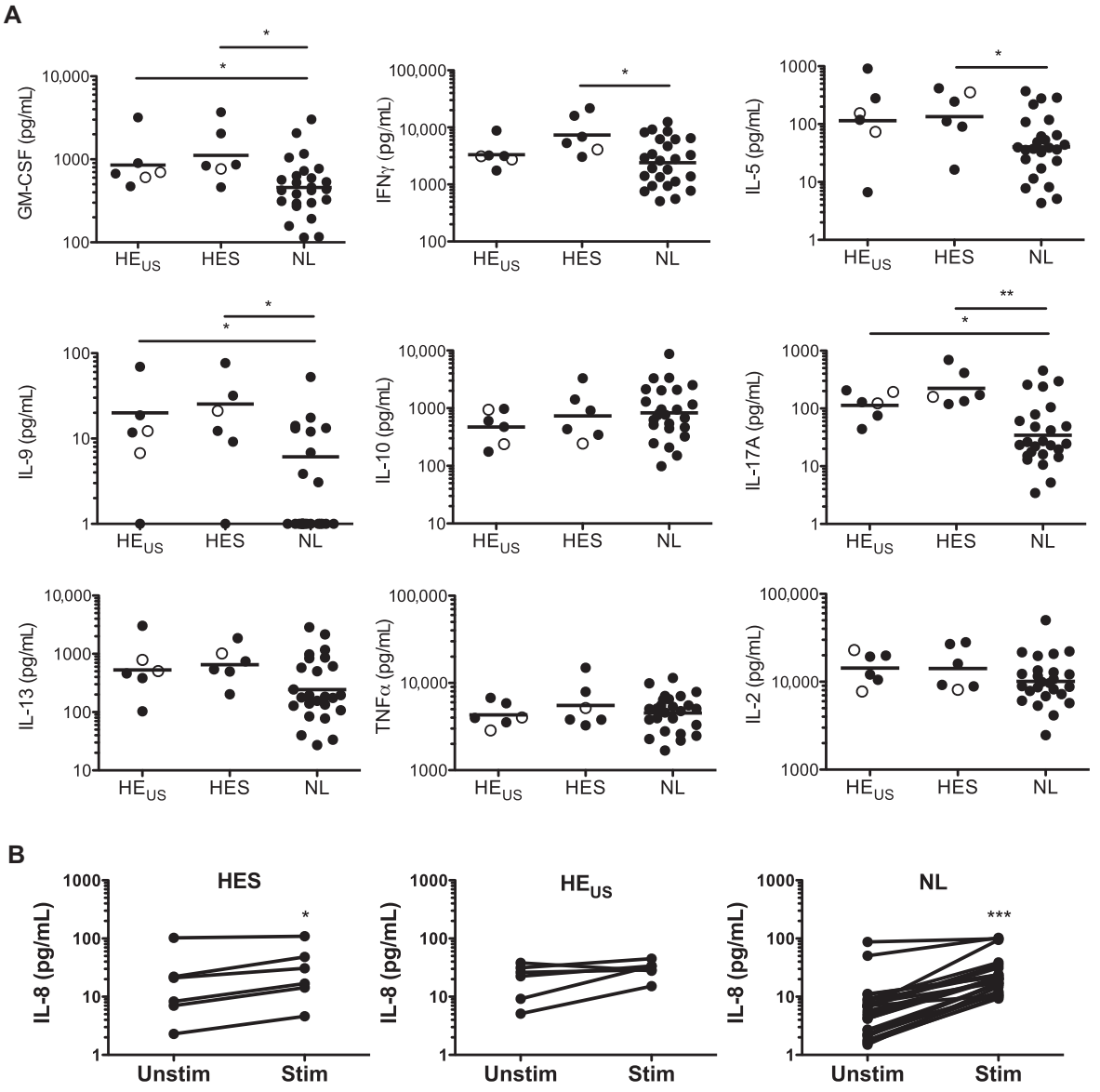

FIG 5. Cytokine and chemokine production by PBMCs. **A,** Levels of GM-CSF, IFN-γ, IL-5, IL-9, IL-10, IL-17A, IL-13, TNF-α and IL-2 in supernatants from PMA/ionomycin-stimulated PBMCs from subjects with HE_{US} (n = 6) and untreated HES (n = 6) and normal controls (n = 25). Values are represented as stimulated minus unstimulated cytokine levels measured in supernatants collected after 6 hours in culture. Each *symbol* represents data from an individual subject. The *horizontal bar* denotes the GM. *Open circles* represent subjects with a clonal and/or aberrant T-cell population. $*P < .05$, $**P < .01$, and $***P \leq .001$, Mann-Whitney test. **B,** Change in IL-8 levels in PBMC supernatants following PMA/ionomycin stimulation. $*P < .05$, $***P < .0001$, Wilcoxon signed-rank test. *NL*, Normal; *Stim*, stimulated; *Unstim*, unstimulated.

reviews of HES,[1-3] although little is known about the prevalence, clinical characteristics, and prognosis of such patients in the absence of therapy. In the present study, 8 (3.8%) of 210 subjects referred for unexplained (AEC >1500/μL) (and 22% of asymptomatic *FIP1L1/PDGFRA*-negative subjects on no therapy at the time of referral) met the proposed diagnostic criteria for HE_{US}. Three additional subjects (HES subjects 7, 13, and19; see the Online Repository) were, in fact, untreated for more than 5 years following the documentation of AEC > 1500/μL; however, all 3 had clinical signs and symptoms at the time that eosinophilia was first noted, which progressed over time, requiring drug treatment in 2 subjects and esophageal dilatation in the third subject. *PDGFRA*-positive subjects do occasionally present before the development of clinical manifestations but were excluded from the study analysis, because treatment delay would have been unethical in view of the poor prognosis of such patients before the availability of effective therapy with imatinib mesylate.[8] Among the 15 subjects excluded because of *PDGFRA* positivity, 1 was, in fact, asymptomatic at the time that eosinophilia was first noted. Treatment was initiated 18 months later when testing returned positive for *FIP1L1/PDGFRA*.

With the exception of a complete absence of signs or symptoms attributable to eosinophilia, subjects with HE_{US} were remarkably similar to those with HES (Table I). Of particular note, neither

eosinophil counts nor surface markers of eosinophil activation were significantly different between the 2 groups. Serum levels of soluble IL-2 receptor, which have been associated with disease activity in HES,[9] were also comparably elevated in subjects with HE_{US} and HES than in normal controls. Although serum IgE levels of more than 1000 IU/mL were seen only in subjects with HES and might be useful in identifying individuals at risk for the development of clinical manifestations, no clinical predictors of a benign outcome were identified.

None of the subjects with HE_{US} and only 2 of the subjects with HES included in the analysis had elevated serum B12 levels or other features suggestive of a primary myeloproliferative disorder, although this low prevalence was expected because of the exclusion of subjects with *PDGFRA*-positive disease from the analysis. Clonal and/or aberrant T-cell populations consistent with lymphocytic variant HES were present in comparable frequencies in subjects with HE_{US} and HES (50% and 29%, respectively) and were consistent with the reported prevalence of 17% to 43% in published series of patients with HES.[6,10,11] Serum TARC levels were also elevated in a high percentage of subjects in both groups, consistent with a predominance of lymphocyte-driven eosinophilia.[4,7,11]

The cytokine and chemokine profiles in serum and PBMC supernatants were generally similar between subjects with HE_{US} and HES, with increased levels of cytokines associated with eosinophilia and eosinophilic disorders, including IL-5, GM-CSF, IL-9, and IL-17A, in both groups as compared to normal subjects. GM serum levels of IL-13, a key cytokine in the pathogenesis of eosinophilic disorders,[12,13] were significantly elevated only in the HES group compared to normal controls, consistent with the increased serum IgE levels in this group as compared to those in subjects with HE_{US}. GM net levels of IL-13 in PBMC supernatants were also increased in subjects with HES as compared to controls, although this did not reach statistical significance (651 vs 244 pg/mL, respectively; $P = .068$, Mann-Whitney *U* test). Although the potential role of relatively diminished IL-13 and IgE responses in the lack of clinical manifestations in HE_{US} remains to be confirmed in a larger cohort, recent data from clinical studies of lebrikizumab, an mAb to IL-13, in patients with asthma suggest that IL-13 promotes eosinophil migration to tissues without an appreciable effect on peripheral blood eosinophilia,[13] consistent with this hypothesis. Significant elevations in the serum levels of IL-10, TNF-α and IFN-γ were also seen only in subjects with HES as compared to controls. Although levels of these cytokines were comparable between subjects with HE_{US} and HES, a role for inflammatory and T_H1 cytokines in determining the clinical manifestations of eosinophilia cannot be entirely excluded because of the small size of the HE_{US} study group.

The present study has 2 significant and interrelated limitations: the paucity of subjects with HE_{US} and the retrospective design, with identification of subjects with HE_{US} only after they had been followed off therapy for more than 5 years. HES is an extremely rare disorder estimated to affect 0.3 to 6.3/100,000 people in the United States.[14] Consequently, even a large referral center is unlikely to evaluate sufficient patients with presumed HES in any given year to support a prospective study of HE_{US}. Although it is likely that HE_{US} is much more common than the current study suggests, because asymptomatic patients with eosinophilia (AEC >1500/μL) may not be referred for further evaluation by their primary providers, the true prevalence of HE_{US} is unknown. This is further complicated by the fact that current *International Classification of Diseases, Ninth Revision* codes do not distinguish between HES and HE_{US}.[15]

Despite these limitations, the present study clearly demonstrates that a subset of patients with persistent eosinophilia (AEC > 1500/μL) does not develop clinical manifestations of HES in the absence of therapy over a prolonged period of time (up to 31 years). Furthermore, these asymptomatic eosinophilic subjects with HE_{US} were indistinguishable at presentation from patients with untreated symptomatic idiopathic or lymphocytic variant HES on the basis of demographics, routine laboratory testing, measures of eosinophil activation, or cytokine profiles. Although these data suggest that completely asymptomatic patients with AEC > 1500/μL can be followed closely without specific treatment for the eosinophilia, it is important to note that all of the subjects in this study underwent comprehensive evaluation at the time of presentation, were followed with monthly eosinophil counts for at least 1 year, and continue to be assessed yearly for end-organ manifestations attributable to eosinophilia. A multicenter prospective study is clearly needed to better assess the risk of and predisposing factors for progression to HES in patients with HE_{US}.

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Clinical implications: A subset of patients who present with unexplained marked eosinophilia (AEC > 1500/μL) and no clinical manifestations attributable to eosinophilia appear to have a benign prognosis and can be followed closely without therapy.

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METHODS

Assessment of T-cell phenotype

Whole blood was stained with CD45-APC-efluor 780 (eBioscience, San Diego, Calif), CD14-fluorescein isothiocyanate (FITC), CD3-phycoerythrin-Cy5 (BD Biosciences, San Jose, Calif), CD-4 PerCP, and CD8-APC (Invitrogen). Irrelevant, directly conjugated, murine IgG_1 was used to ascertain background staining. Lymphocytes were identified by a combination of light scatter and antibody staining (bright CD45-positive and CD14-negative). Aberrant T cells were identified within the lymphocyte gate on the basis of CD3, CD4, and CD8 expression. Additional staining with CD5-FITC and CD7-FITC was performed in samples with 1.5% or more CD3-$CD4^+$ cells. Data were collected on a FACSCanto flow cytometer by using Diva software (BD Biosciences) and exported to FCS Express software (De Novo Software, Los Angeles, Calif) for further analysis.

Surface expression of eosinophil activation markers

Whole blood was stained with CDw125-phycoerythrin (clone A14; BD Biosciences), CD25-FITC, CD9-FITC, CD69-FITC, and CD16-phycoerythrin (BD Biosciences). Irrelevant, directly conjugated, murine IgG_1 was used to ascertain background staining. CD9 was used as a positive control for eosinophils. Samples were collected on a FACSCanto II flow cytometer by using Diva software (BD Biosciences). Eosinophils were separated from granulocytes by their characteristic high-side scatter and dim staining for CD16. Percent positive for each surface molecule was ascertained by using a FITC-conjugated subclass control and setting a marker so that more than 98% of the control was defined as negative.

TABLE E1. Clinical manifestations of subjects with HES

Subject	AEC/mm^3 at presentation to the National Institutes of Health	End-organ manifestations	HES therapy* (chronological order)	Years of eosinophilia before treatment
1	2,999	Pruritic skin rash	Prednisone	0.7
2	4,884	Urticaria, pruritus, fatigue, night sweats	Prednisone	0.5
3	4,136	Myalgias, fatigue, weight loss	Prednisone	0.5
4	6,878	Cough, pulmonary infiltrates/fibrosis	Unknown†	0.5
5	45,990	Sinusitis, testicular pain, lymphadenopathy, fatigue, malaise	Prednisone	0.1
6	5,111	Necrotic skin ulcers, lymphadenopathy	Prednisone, IFN-α	0.1
7	2,062	Asthma, sinusitis, urticaria, facial rash, paresthesias, Raynaud's phenomenon, fatigue, malaise	Prednisone, hydroxyurea, mepolizumab	10
8	3,510	Urticaria, fatigue, dyspnea, pulmonary infiltrate, malabsorption	Prednisone	1.75
9	6,420	Fever, fatigue, splenomegaly, polycythemia	Prednisone, imatinib, IFN-α, mepolizumab, hydroxyurea	0.4
10	10,080	Arthralgias, myalgias, angioedema, pruritic skin rash, abdominal pain, fatigue	Prednisone, hydroxyurea, IFN-α, imatinib, reslizumab, cyclosporine, IVIG, mycophenolate mofetil, mepolizumab, lenalidomide, cytoxan, bone marrow transplant	0.5
11	4,160	Cough, fever, diarrhea, night sweats, eosinophilic hepatitis	Prednisone	3
12	2,901	Myalgias, arthritis, flushing, splenomegaly, fatigue	Prednisone, hydroxyurea, imatinib, IFN, mepolizumab, methotrexate, cyclophosphamide, dasatinib, mycophenolate mofetil, alemtuzumab	2
13	1,669	Gastroesophageal reflux, dysphagia	None	NA†
14	3,016	Asthma	Imatinib, prednisone, mepolizumab	1.5
15	2,310	Pruritic rash, bullous skin lesions, lymphadenopathy, episcleritis	Unknown‡	NA
16	2,334	Myalgia, nausea, vomiting, fever, eosinophilic hepatitis	Prednisone	4
17	1,654	Diarrhea, abdominal pain	Prednisone	1
18	1,640	Asthma, sinusitis, pulmonary infiltrates, myocarditis	Prednisone	4
19	2,208	Myalgias, arthralgias, fatigue, weight loss, pulmonary infiltrates	Prednisone	12
20	3,550	Myalgias, arthralgias, night sweats, fatigue, pruritic skin rash, angioedema	Unknown‡	NA
21	7,080	Asthma, sinusitis, urticaria, skin rash, alopecia	Prednisone, hydroxyurea, IFN-α, omalizumab, IVIG	0.2
22	13,357	Cough, pulmonary fibrosis, pruritic rash	Prednisone, imatinib	3
23	3,674	Sinusitis, asthma, chronic cough, pulmonary infiltrates	Prednisone, mepolizumab	On steroids at the time of presentation
24	7,797	Nausea, right upper-quadrant pain, myalgias, arthralgias, fevers, headache, malaise, fatigue, pruritic rash, eosinophilic hepatitis	Prednisone, imatinib, mepolizumab	0.1
25	6,070	Asthma, eosinophilic enteritis with protein-losing enteropathy	Prednisone, mepolizumab	0.1
26	2,177	Abdominal pain, diarrhea, pulmonary infiltrates	Prednisone, imatinib	4
27	3,780	Angioedema, pulmonary infiltrates, neuropathy	Prednisone, mepolizumab	2
28	1,530	Asthma, pulmonary infiltrates, sinusitis, paresthesias	Prednisone	0.2

EGD, Esophagogastroduodenoscopy; *IVIG*, intravenous immune globulin; *NA*, not applicable.

*No subjects were on therapy at the time of initial evaluation at the National Institutes of Health. Subject 23 had previously been on prednisone therapy.

†Initial EGD performed for reflux symptoms at the time eosinophilia was noted showed no pathology; the patient was lost to follow-up until 15 years later, patient developed dysphagia and a diagnosis of eosinophilic esophagitis with stricture formation was confirmed; the patient underwent dilatation, but does not wish to be treated systemically.

‡Patient lost to follow-up.