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Anti-IL-5 (mepolizumab) therapy reduces eosinophil activation *ex vivo* and increases IL-5 and IL-5 receptor levels

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Abstract

Background—Anti-IL-5 might be a useful therapeutic agent for eosinophilic disorders, yet its immunologic consequences have not been well characterized.

Objective—We sought to characterize the hematologic and immunologic effects of anti-IL-5 in human subjects.

Methods—The effects of 3-month infusions of mepolizumab were assessed in 25 patients with a variety of eosinophilic syndromes. Samples with increased IL-5 levels after therapy were analyzed by using size exclusion filtration. Immunoreactive IL-5 fraction and plasma samples were subsequently precipitated with saturating concentrations of protein A/G.

Results—Twenty-three patients responded to anti-IL-5 therapy with a decrease in blood eosinophil counts and a reduced percentage of CCR3⁺ cells by 20- and 13-fold, respectively ($P < .0001$). Responsiveness was not related to the levels of baseline plasma IL-5 or the presence of *FIP1L1-PDGFR* fusion gene. Persistently decreased blood eosinophilia remained for 3 months after final infusion in 76% of subjects. Therapy was associated with a large increase in blood IL-5 levels, likely because of a circulating IL-5/mepolizumab complex precipitated with protein A/G, a significant increase in eosinophil IL-5 receptor α expression, and increased percentage of CD4⁺ and CD8⁺ cells producing intracellular IL-5 ($P < .05$). Additionally, anti-IL-5 therapy decreased eotaxin-stimulated eosinophil shape change *ex vivo*.

Conclusions—Anti-IL-5 therapy induces a dramatic and sustained decrease in blood eosinophilia (including CCR3⁺ cells), decreased eosinophil activation, and increased circulating levels of IL-5 in a variety of eosinophilic disorders. Increased levels of IL-5 receptor α and lymphocyte IL-5 production after anti-IL-5 therapy suggest an endogenous IL-5 autoregulatory pathway.

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Keywords

Anti-IL-5; cytokines; eosinophilia; esophagitis; hypereosinophilic; IL-5; inflammation; mepolizumab

IL-5 regulates multiple major eosinophil functions, including cellular proliferation, mobilization from the bone marrow into the peripheral circulation, maturation, activation, tissue recruitment, survival, and priming to stimulating agents.^{1,2} Overproduction of IL-5 has been reported in patients with a variety of eosinophil-associated disorders, including hypereosinophilic syndrome (HES),^{3,4} lymphoma,⁵ eosinophil-associated gastrointestinal disorders (EGID),^{6,7} and eosinophilic esophagitis (EE).^{8,9} As such, IL-5 is a potential therapeutic target for the treatment of eosinophil-mediated diseases. Indeed, inhibition of IL-5 has dramatic effects on eosinophils, as demonstrated by analysis of *IL-5* gene-deficient mice (or mice treated with neutralizing antibodies) that have impaired development of antigen-induced eosinophilia after parasite and allergen exposure.^{10–12} This has prompted the development of humanized antibodies against IL-5 for the treatment of asthma. Although early clinical trials revealed that anti-IL-5 was safe and effective in decreasing blood eosinophil levels in asthmatic patients and healthy individuals, clinical trials in patients with asthma revealed no major improvement in clinical parameters, likely because lung eosinophilia was reduced by only approximately 2-fold by anti-IL-5 therapy.^{13–15} Although anti-IL-5 might benefit some patients with asthma and lung remodeling,¹⁶ recent attention has focused on the potential utility of anti-IL-5 in treating other eosinophil-associated diseases, such as HES and EE. Preliminary data in patients with HES and EE treated with anti-IL-5 have revealed promising results, including improvements in clinical parameters and levels of tissue eosinophilia.^{17–20} The first randomized, double-blind, placebo-controlled trial of patients with HES was recently conducted and aimed at demonstrating the ability of anti-IL-5 to decrease prednisone dependence in HES not associated with the constitutively activated tyrosine kinase *FIP1L1*-platelet-derived growth factor receptor α (*FIP1L1*-PDGFRA).²¹ Impressively, this international trial revealed a striking ability of anti-IL-5 to decrease prednisone doses, decrease blood eosinophilia, and maintain clinical stability compared with placebo. Surprisingly, an in-depth evaluation of the hematologic and immunologic effects of anti-IL-5 in human subjects has not been conducted (even in asthmatic individuals). Here we report such results after an open-label trial of anti-IL-5 (mepolizumab) in 25 subjects with diverse eosinophilic disorders.

METHODS

Protocol and subject characteristics

Inclusion and exclusion criteria—Twenty-five subjects (13 male and 12 female subjects 18–57 years of age) were enrolled in an open-label phase I/II trial designed to assess the safety of mepolizumab and to delineate the effect of neutralizing endogenous IL-5 activity in patients with eosinophilic disorders, as previously described.^{18,20}

Subject characteristics—Of the 25 subjects enrolled, 18 were diagnosed with a diverse set of HESs, as defined by a recent workshop (Table I).²² Six subjects were diagnosed with EE. Of these subjects, 4 subjects with EE and 3 subjects with HES had participated in a previous study that evaluated peripheral blood and tissue eosinophil counts in response to anti-IL-5 therapy, but they were not evaluated for immunologic changes.^{18,20} One other subject was diagnosed with EGID only. No study subject had previously received mepolizumab; in total, there were 7 overlap subjects within other mepolizumab clinical trials.

Protocol—This study was conducted with the approval of the institutional review board and the Food and Drug Administration, as well as the informed consent of participating subjects

(clinicaltrials.gov, NCT00266565). Subjects received 3 intravenous infusions of the anti-IL-5 medication mepolizumab (GlaxoSmithKline, Research Triangle Park, NC) at a dose of 10 mg/kg (maximum, 750 mg) at weeks 8, 12, and 16. During weeks 0 through 8, those subjects with a history of peripheral blood eosinophilia and eosinophil counts of less than 750 cells/mm³ had their antieosinophil therapy (glucocorticoids) tapered until their absolute eosinophil levels increased 2-fold over baseline, and/or their eosinophil count increased to greater than 750 cells/mm³.

All subjects received 3 mepolizumab infusions at the same dosage. Cohort A (n = 14) subjects received mepolizumab with no modification to their currently prescribed immunosuppressant or antieosinophil medications. Cohorts B (n = 6) and C (n = 5) subjects received mepolizumab and had their immunosuppressant or antieosinophil medications reduced by 25% or 50%, respectively, after the second mepolizumab infusion (at week 12). Blood was collected at week 8 (before the first infusion) and at week 20 (4 weeks after the third and final infusion) for analysis and assaying. Fig 1, A, depicts the protocol followed. Twenty-three of the 25 subjects completed the trial; subject 12 withdrew because of a lack of therapy efficacy, and subject 13 withdrew because of concern about the potential risks of treatment.

Blood from 27 donors without an eosinophilic disorder was used as a control to assess the levels and ranges of the different assays used in this study. Peripheral blood eosinophil counts were measured as previously reported.²⁰ Response to therapy was defined as a statistically significant reduction in peripheral blood eosinophil counts. RT-PCR for detection of *FIP1L1-PDGFR* fusion gene was performed by using methods previously reported.²³

Plasma IL-5

Plasma IL-5 levels were determined by using the OptEIA ELISA kit (BD Biosciences Pharmingen, San Diego, Calif) and also by using the IL-5 Quantikine ELISA Kit (R&D Systems, Minneapolis, Minn) at week 20 to confirm the high IL-5 levels observed in some of the subjects after mepolizumab therapy, according to the manufacturer's instructions. The detection limit of both ELISA kits was 7.8 pg/mL. The detection limit value was used for data of less than 7.8 pg/mL to calculate IL-5 values; statistical analysis was performed with a paired nonparametric test (Wilcoxon signed-rank test).

Blood samples that had a high IL-5 level at week 20 were compared with a control IL-5 standard sample from the OptEIA ELISA kit to determine free and bound plasma IL-5 by using cellulose membrane filtration tubes for a molecular weight of 100 kd (Microcon YM-100 centrifugal filter device; Millipore and Amicon Corp, Bedford, Mass), according to the manufacturer's instructions. Both the filtrated lower-chamber fraction (low molecular weight, free IL-5) and the upper-chamber fraction (unfiltered high molecular weight, bound IL-5, diluted×4 in PBS) were analyzed for IL-5 levels (OptEIA ELISA kit). Representative samples (120 µL) from the upper chamber were subsequently incubated with 50 µL of protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) overnight at 4°C and centrifuged (3500 rpm for 5 minutes), and the supernatant was analyzed for IL-5.

Original plasma samples with high IL-5 levels were precipitated with a saturating concentration (200 µL) of protein A/G PLUS-Agarose and tested again by using the same protocol to reassess the presence of a high-molecular-weight bound IL-5 immunocomplex at week 20. A representative sample was used for a titration (0–500 µL of protein A/G) dose-response curve to calculate the saturation plateau so that no further detection of IL-5 was observed in the supernatant of the sample. Two samples (from subjects nos. 7 and 24) were diluted previously because of the high IL-5 concentration levels and then precipitated with 200 µL of protein A/G PLUS-Agarose.

Immunofluorescence analysis

IL-5 receptor (IL-5R) α CDw125, CCR3⁺ cells, peripheral blood lymphocyte subsets, and intracellular cytokines were measured by means of flow cytometry, as previously described.²⁰ The samples (100 μ L of whole blood) were stained with 0.25 μ g of mAbs to CCR3–fluorescein isothiocyanate (FITC; R&D Systems) and CDw125–phycoerythrin (PE; BD Immunocytometry Systems, San Jose, Calif) and with appropriate isotype-matched control antibodies (BD) for 20 minutes at room temperature. Red cells were lysed with FACSlyse (BD), and leukocyte cell pellets were then fixed (1% paraformaldehyde) and analyzed with a FACSCalibur flow cytometer (BD) by using a 4-decade log scale. CCR3 positivity and side-scatter properties distinguished the eosinophils from peripheral blood neutrophils and lymphocytes.^{1,24} Two different lymphocyte subsets were stained with either surface mAbs (1) anti-CD3-FITC, anti-CD8-PE, anti-CD45-peridinin chlorophyll-protein (PerCP), and anti-CD4-allophycocyanin (APC) or (2) anti-CD3-FITC, anti-CD16⁺CD56-PE, anti-CD45-PerCP, and anti-CD19-APC. Results are reported as the percentage of positive cells per lymphocyte population. Data were analyzed with MultiSET software (BD).

Intracellular cytokine production

Intracellular cytokines (IL-4, IL-5, IL-13, IFN- γ , and TNF- α) were analyzed from whole-blood samples by using 4-color flow cytometry, as previously described.^{25–27} Cytokine production was stimulated by incubating 500 μ L of whole blood diluted 1:1 with RPMI 1640 media (Mediatech, Inc, Herndon, Va), phorbol 12-myristate 13-acetate (20 ng/mL), and ionomycin (1 μ g/mL; Sigma-Aldrich, St Louis, Mo) for 4 hours at 37°C in a 5% CO₂ atmosphere. Activation was carried out in the presence of brefeldin-A (10 μ g/mL; Sigma-Aldrich). Unstimulated samples were treated only with brefeldin-A and served as a negative control. Successful lymphocyte activation was confirmed by comparing CD69 staining of activated (96% CD69⁺ cells) and nonactivated cells. The activated cells were stained at room temperature for 20 minutes with the surface mAbs anti-CD3-FITC and anti-CD8-PerCP (BD Immunocytometry Systems) and either anti-CD56-PE (BD) or anti-CD56-APC (Immunotech, Brea, Calif), fixed, and permeabilized with Intraprep reagent (Immunotech Beckman Coulter, Miami, Fla). Cells were then stained with antibodies to the intracellular cytokines anti-IFN- γ -APC, anti-TNF- α -APC (BD Pharmingen), anti-IL-4-PE, anti-IL-5-PE, and anti-IL-13-PE (BD) or their corresponding isotype controls; washed and resuspended in 1% paraformaldehyde; and analyzed by means of flow cytometry with Cell Quest Pro software (BD). Staining was performed on the same day on control samples to control for day-to-day variability and allow comparative analysis between individuals.

Peripheral blood cell purifications and assays

PBMC cytokine secretion—PBMCs were incubated with 25 μ g/mL PHA (Sigma-Aldrich) for 48 hours at 37°C in a 5% CO₂ atmosphere, with 10% pooled human serum (Integen, Milford, Mass) added after 1 hours. Cytokine secretions in culture supernatants were harvested and frozen at –80°C until analyzed by means of ELISA. The cytokines IL-4, IL-5, IL-10, GM-CSF, and IFN- γ in culture supernatants were measured by using an OptEIA ELISA kit (BD Biosciences), and IL-13 was measured by using the Human IL-13 Immunoassay Kit (BioSource International, Camarillo, Calif).

Granulocyte purification and shape change with flow cytometry—Blood samples were prepared for quantitative eosinophil shape-change response to chemoattractant agonists by using the gated autofluorescence/forward scatter assay by means of flow cytometry, as previously described.^{24,28}

Statistical analysis

Statistical analyses were performed with GraphPad Prism for Windows version 4.0 (GraphPad Software, San Diego, Calif). Normally distributed data were expressed as means $\pm$ SD. Data that did not show a normal distribution were presented as medians with 25% and 75% interquartiles. Differences before and after treatment were tested by using parametric (paired and unpaired Student *t* tests) or nonparametric (Wilcoxon signed-rank test) tests, as appropriate. Statistical significance was defined as a *P* value of less than .05.

RESULTS

Effect of anti-IL-5 therapy (mepolizumab) on peripheral blood eosinophil levels

Subjects (Table I) were observed, stabilized, or both for 8 weeks, subsequently infused with 3 doses of intravenous mepolizumab at 4-week intervals (weeks 8, 12, and 16), and monitored thereafter for an additional 12 weeks (Fig 1, A). Mepolizumab therapy was generally well tolerated (Table I).

During the first 8 weeks, the mean eosinophil level ranged from 1183 ± 2073 cells/mm³ at baseline to 1400 ± 2179 cells/mm³ (mean $\pm$ SD, *P* = .15). After mepolizumab therapy, a marked and sustained decrease in blood eosinophil levels occurred in 23 (92%) of 25 subjects. Subjects without increased blood eosinophil levels before therapy but with tissue eosinophilia also responded with an observed decrease in blood eosinophil levels after mepolizumab therapy (Table I and Fig 1, B). One of 2 resistant subjects (subject 12) displayed clear nonresponsiveness because blood eosinophilia remained increased despite 2 mepolizumab treatments. Subject 21 displayed no decrease in blood eosinophilia; however, his initial eosinophil count was relatively low (120 cells/mm³), and he was able to reduce his corticosteroid treatment by 50% with no resulting eosinophilia (Table I). Overall, mepolizumab therapy effectively reduced peripheral blood eosinophilia from 1400 ± 2179 to 64 ± 54 cells/mm³, a 21.8-fold decrease (*P* < .0001; Fig 1, C), and the percentage of eosinophils in the blood from $15\% \pm 16\%$ to $1\% \pm 1\%$ (*P* < .0001; Table I and Fig 1, D). Sixteen (76.2%) of 21 subjects displayed significantly decreased peripheral blood eosinophil levels for 3 months after the final mepolizumab infusion (*P* = .0002; Fig 1, E). This sustained decrease in blood eosinophil levels occurred mostly in cohort A subjects (from 1573 ± 2778 to 83 ± 59 cells/mm³, a 19-fold decrease; *P* < .0005). Partial to complete restoration of peripheral blood eosinophil levels observed at week 28, defined as the increase in eosinophil number by at least 50% of the eosinophil levels observed at week 8, was observed in 7 (33.3%) of 21 subjects (2 [18.2%] of 11 from cohort A, 2 [40%] of 5 from cohort B, and 3 [60%] of 5 from cohort C), although eosinophils were in the range levels measured previous to therapy (weeks 0–8) in most of the subjects. Of note, 1 subject in each cohort (3 [14.3%] of 21 subjects) experienced an increase of greater than 100% in eosinophil levels at week 28 compared with levels at week 8, an increase from 1056 ± 413 cells/mm³ ($10\% \pm 1\%$) to 2496 ± 1918 cells/mm³ ($18\% \pm 10\%$; mean $\pm$ SD), but only subject 23, who had glucocorticoid therapy gradually reduced by 50% after the second mepolizumab infusion, had eosinophil levels substantially higher at week 28 compared with levels at weeks 0 through 8 (4710 vs 920 cells/mm³ [range, 50–1410 cells/mm³], respectively). The only subject (no. 16) with a positive test result for *FIP1L1-PDGFR*A fusion gene (and who did not respond to prior therapy with imatinib [Table I]) displayed a response to mepolizumab treatment comparable with that of the rest of the subjects with negative test results for the gene. Analysis of patients who started with eosinophil levels of greater than 1500 cells/mm³ revealed that mepolizumab decreased eosinophil levels from 2566 ± 1305 cells/mm³ ($31\% \pm 10\%$) to 50 ± 29 cells/mm³ ($1\% \pm 0\%$), a 52-fold decrease (*P* = .03, *n* = 6).

No significant change in white blood cell count or percentage of blood lymphocytes was observed during the study (Fig 1, D).

Plasma IL-5 and anti-IL-5 therapy (mepolizumab)

Plasma IL-5 levels were increased in 3 of 24 subjects before mepolizumab therapy. Interestingly, these subjects (nos. 6, 13, and 18) had HES with associated EGID and had plasma IL-5 levels of 15, 24, and 30 pg/mL, respectively (detection limit, 7.8 pg/mL). Plasma levels of IL-5 did not correlate with responsiveness to mepolizumab therapy (Table I).

Of note, a significant increase in detectable plasma IL-5 levels was observed in 15 subjects after mepolizumab therapy ($P < .0001$; Fig 2, A); paired analysis is shown in Fig 2, B.

We hypothesized that increased levels of IL-5 might reflect the presence of a relatively long-lived IL-5/mepolizumab complex. To address this, we examined the molecular size of immunoreactive IL-5, hypothesizing that free IL-5 would pass through an exclusion filter of 100 kd. We found no detection of low-molecular-weight IL-5 (lower chamber) at week 20 (4 weeks after the final mepolizumab infusion), but immunoreactive IL-5 was completely detected in the higher-molecular-weight fraction of a Microcon filter (upper chamber; Fig 3, A). As a control, we examined the distribution of recombinant IL-5 after similar filtration. As expected, in contrast to samples collected after mepolizumab treatment, recombinant IL-5 was completely present in the low-molecular-weight fraction (Fig 3, A). To further identify the nature of the IL-5 complex, representative samples from the high-molecular-weight fraction were precipitated with protein A/G (Fig 3, B), resulting in marked precipitation of IL-5. As a control, the ability of protein A/G to bind IL-5 normally produced by PBMCs was assessed. As shown in Fig 3, B, PBMC-derived IL-5 was not precipitated by protein A/G. Protein A/G induced a concentration-dependent decrease in immunoreactive IL-5 (Fig 3, C). Importantly, cumulative data from all subjects' samples before protein A/G precipitation revealed that high IL-5 levels at week 20 were reduced to undetectable levels after precipitation with saturating amounts of protein A/G (Fig 3, D).

Effect of anti-IL-5 therapy (mepolizumab) on peripheral blood CCR3⁺ cells

Overall, a 13-fold decrease in the percentage of peripheral blood CCR3⁺ cells was observed after mepolizumab therapy in all subjects (Fig 4, A) and in a paired analysis ($P < .001$; Fig 4, B). Cohort A subjects' peripheral blood percentage of CCR3⁺ cells decreased from $13.6\% \pm 17\%$ to $1.2\% \pm 1\%$, an 11-fold decrease, after mepolizumab therapy (mean $\pm$ SD; $P = .0005$). Similar trends of an 18-fold decrease ($15\% \pm 12\%$ to $0.8\% \pm 0.4\%$) and a 14-fold decrease ($8.8\% \pm 10\%$ to $0.6\% \pm 0.4\%$) were observed in cohort B and C subjects, respectively.

Effect of anti-IL-5 therapy (mepolizumab) on the peripheral blood eosinophil cell-surface markers CCR3 and IL-5R α

Mepolizumab therapy significantly increased the mean channel of fluorescence (mcf) levels of IL-5R α in the entire group (Fig 4, C) and in a paired analysis ($P = .002$; Fig 4, D). Ten (90.9%) of 11 subjects in cohort A had significantly increased mcf levels of IL-5R α (173 ± 20 to 204 ± 40) after mepolizumab treatment (mean mcf $\pm$ SD; $P = .016$). A similar trend was observed in 3 of 4 paired subjects in both cohorts B and C (data not shown). In contrast, the mcf of CCR3 showed no significant changes in expression levels per eosinophil, being 324 ± 96 and 330 ± 88 (mean mcf $\pm$ SD) before (week 8) and after (week 20) mepolizumab treatment, respectively.

Peripheral blood lymphocytes, lymphocyte subset populations, and intracellular cytokine production

Mepolizumab therapy did not change peripheral blood leukocytes, total lymphocytes (absolute numbers and percentage), or lymphocyte subset counts (Fig 1, D, and see Fig E1 in the Online Repository at www.jacionline.org).

Intracellular cytokine (IL-4, IL-5, IL-13, IFN- γ , and TNF- α) production by CD4⁺ and CD8⁺ cells was measured before and 1 month after the final mepolizumab treatment. There was a significant increase in the percentage of both CD4⁺ cells (from 3.1 ± 2.8 to 7.6 ± 7 , mean $\pm$ SD; $P < .05$) and CD8⁺ cells (from 2.0 ± 1.3 to 8.4 ± 8.9 , mean $\pm$ SD; $P < .05$) positive for intracellular IL-5 in 8 of 12 paired subjects and 7 of 12 paired subjects, respectively, after mepolizumab therapy (Fig 5, A–D). In cohort A, 4 of 5 paired subjects had an increased number of CD4⁺ cells producing intracellular IL-5, and 3 of 5 paired subjects had an increased number of CD8⁺ cells producing intracellular IL-5. In cohort B, 3 of 3 paired subjects had an increased number of CD4⁺ cells producing intracellular IL-5, and 2 of 3 paired subjects had an increased number of CD8⁺ cells producing intracellular IL-5. In cohort C, 1 of 4 paired subjects had an increased number of CD4⁺ cells producing intracellular IL-5, and 2 of 4 paired subjects had an increased number of CD8⁺ cells producing intracellular IL-5. There were no statistically significant changes for intracellular IL-4, IL-13, IFN- γ , or TNF- α production by CD4⁺ and CD8⁺ cells after mepolizumab therapy (see Fig E2 in the Online Repository at www.jacionline.org).

Cytokine production by PHA-stimulated PBMCs

There was no statistically significant change in IL-4, IL-5, IL-10, GM-CSF, or IFN- γ production by PHA-stimulated PBMCs after mepolizumab therapy (see Fig E3 in the Online Repository at www.jacionline.org). A modest increase of IL-13 secretion (from 1839 ± 1381 to 2609 ± 2070 pg/mL, mean $\pm$ SD; $P = .0221$) was observed in 13 (65%) of 20 paired subjects after mepolizumab therapy (see Fig E3, G). In cohort A, 8 (73%) of 11 paired subjects had significantly increased IL-13 secretion after mepolizumab therapy (see Fig E3, H; $P = .018$). A similar trend toward increased IL-13 secretion after mepolizumab therapy was observed in 3 of 5 paired subjects in cohort B and 2 of 4 paired subjects in cohort C (data not shown).

Effect of anti-IL-5 (mepolizumab) on eosinophil activation

We tested the effect of mepolizumab on eotaxin-induced eosinophil shape change *ex vivo* because this assay has been well studied and only requires a nominal number of eosinophils.^{24,28} Eosinophil shape change in study subjects in response to eotaxin-1, eotaxin-2, and eotaxin-3 was similar to that in control individuals before mepolizumab therapy. Notably, eotaxin-1, eotaxin-2, and eotaxin-3 induced a dose-dependent increase in eosinophil shape change, with a peak effect seen at 10, 30, and 100 ng/mL, respectively, and a plateau was observed between 30 and 100 ng/mL for eotaxin-1 and eotaxin-2 (Fig 6, A–C). After mepolizumab therapy *in vivo*, a significant decrease in eosinophil shape change was observed in response to eotaxin-1 at concentrations of 10 ng/mL (from 34 ± 5 to 23 ± 9 ng/mL), 30 ng/mL (from 38 ± 4 to 24 ± 8 ng/mL), and 100 ng/mL (from 30 ± 11 to 19 ± 6 ng/mL), in response to eotaxin-2 at concentrations of 30 ng/mL (from 39 ± 6 to 19 ± 4 ng/mL) and 100 ng/mL (from 41 ± 7 to 26 ± 8 ng/mL), and in response to eotaxin-3 at a concentration of 100 ng/mL (from 31 ± 10 to 20 ± 8 ng/mL).

DISCUSSION

Eosinophilic disorders are a heterogeneous group of diseases characterized by blood eosinophilia, tissue eosinophilia, or both with end organ damage. Current therapies for these disorders are often unsatisfactory because of their inefficiency to abate the eosinophilia, their toxicity, or both. Herein we report the immunologic and hematologic effects of anti-IL-5 (mepolizumab) in 25 patients with diverse eosinophilic disorders. Our findings establish that (1) mepolizumab is effective in decreasing blood eosinophilia in 92% of subjects by 22-fold; (2) mepolizumab decreases the level of CCR3⁺ cells in the blood by 13-fold; (3) the beneficial effect of mepolizumab is sustained for 3 months in 76% of subjects after the final infusion; (4) a modest recovery of eosinophilia occurred in 33% of subjects 3 months after mepolizumab

therapy unrelated to baseline eosinophilia or IL-5 levels; (5) subjects responded to mepolizumab regardless of whether their plasma IL-5 levels were normal or increased before therapy; (6) mepolizumab therapy allowed concurrent antieosinophil therapy (typically glucocorticoids) to be reduced by 25% to 50%; (7) mechanistic analysis revealed that mepolizumab therapy was associated with a compensatory increase in IL-5R α expression by eosinophils, as well as increased CD4⁺ and CD8⁺ cell IL-5 production, providing evidence for an endogenous autoregulatory pathway; and (8) mepolizumab therapy decreased eosinophil responsiveness to chemokine activation *ex vivo* (as measured by eosinophil shape-change responses to eotaxin).

Only 1 subject (no. 12) clearly did not respond to treatment because eosinophilia remained high after mepolizumab therapy. This subject, with negative results for *FIP1L1-PDGFR*A fusion gene, had undetectable baseline plasma IL-5 levels, lower levels of baseline IL-5R α expression (133 vs 171 $\pm$ 31 mcf), and the highest IL-13 secretion by stimulated PBMCs (5662 pg/mL vs subjects' mean of 1839 $\pm$ 1381 pg/mL).

An interesting finding resulting from this study was the observed increased level of plasma IL-5 after mepolizumab therapy. We hypothesized that the increased level of immunoreactive IL-5 was the consequence of a mepolizumab/IL-5 complex because neutralizing antibodies have been shown to increase the half-life of cytokines *in vivo*.²⁹ Using size exclusion filtration, we demonstrated that immunoreactive IL-5 was completely detectable in a large-molecular-weight fraction (>100 kd) after mepolizumab infusion. IL-5 is normally produced as a homodimer (estimated molecular weight is 52 kd),^{30,31} and indeed, in control experiments both recombinant IL-5 and naturally produced IL-5 were detectable in the low-molecular-weight fraction. To further elucidate the nature of high-molecular-weight immunoreactive IL-5 after mepolizumab therapy, we demonstrated that IL-5 was completely precipitated by means of incubation with protein A/G, thereby providing evidence that its high molecular weight was due to an immunoglobulin/IL-5 complex. Although this complex is likely a mepolizumab/IL-5 complex, without an anti-mepolizumab antibody, we cannot rule out the presence of another immunoglobulin in the complex. In theory, neutralizing anti-cytokine antibodies have the capacity to prolong the half-life and activity of cytokines²⁹; however, the prolonged activity only occurs at low molar antibody/cytokine ratios. In the case of mepolizumab therapy, pharmacologic doses of the drug are present (well above the endogenous IL-5 molar amount), and indeed, the presence of the high-molecular-weight IL-5/immunoglobulin complex correlates with neutralizing activity (decreases of eosinophilia *in vivo*). It remains possible that free IL-5 could be released from the mepolizumab/IL-5 complex as the concentration of the drug decreases; however, levels of the complex did not correlate with the level of blood eosinophilia after the final mepolizumab infusion in the majority of subjects.

We observed increased levels of IL-5R α and lymphocyte IL-5 production after mepolizumab therapy, suggesting the existence of an endogenous IL-5 autoregulatory pathway. In view of other proposed effects of IL-5 on IL-5R expression,³² it remained possible that detection of IL-5R α expression could be directly influenced by the endogenous IL-5 (if IL-5 and anti-IL-5 competed for the same binding site); however, IL-5R α expression was not affected by preincubating eosinophils with a wide concentration range of IL-5 *in vitro* (see Fig E4 in the Online Repository at www.jacionline.org). In addition, the effect on IL-5R α was specific because mepolizumab therapy did not alter eosinophil expression of CCR3, CD69, or CD25. This theory is supported by recent observations in eosinophil-deficient mice that have increased IL-5 levels.³³ Our previous studies have demonstrated increased levels of IL-5 in the plasma of eosinophil lineage-ablated Δ dbl-GATA mice compared with levels in wild-type mice (43.2 $\pm$ 9.5 vs 15.2 $\pm$ 11.3 pg/mL; P = .03; n = 3 experiments; Fulkerson and Rothenberg, unpublished findings). In the present study we could not demonstrate an increase in the secretion of IL-5 by stimulated PBMCs after mepolizumab therapy, even though the secretion of IL-13 was

increased in PHA-stimulated PBMCs, suggesting that other signals might be involved in stimulating the production of IL-5 and IL-13 after mepolizumab treatment. The upregulation of IL-5 production and IL-5R α expression raises concerns that the discontinuation of mepolizumab therapy might be associated with an exaggerated IL-5 pathway. Indeed, a smaller clinical trial with another humanized anti-IL-5 antibody (reslizumab) has suggested that rebound eosinophilia might be related to subsequent overproduction of IL-5.^{19,34} Mepolizumab has a half-life of 13 ± 2 days after intravenous administration,³⁵ but its biologic activity can be longer, as observed by the significant decrease in peripheral blood eosinophil levels that persisted for 3 months in 76% of subjects after the final mepolizumab infusion. Interestingly, in 33% of subjects, a partial to complete recovery of peripheral blood eosinophil levels (an increase of 50% or greater) was observed at week 28, approaching levels observed at week 8; an even greater effect on eosinophil levels was observed when corticosteroid therapy was decreased. Significant differences between reslizumab and mepolizumab might be relevant to the risk of a rebound effect because reslizumab (IgG4 antibody) was infused at a lower dose (0.3 and 1 mg/kg) than mepolizumab (IgG1 antibody, infused at 10 mg/kg), and the molar antibody/cytokine concentration of mepolizumab might affect its ability to release IL-5 at lower doses. It is worth noting, however, that the increase in eosinophil levels observed at week 28 was fairly modest, suggesting, perhaps, the normal variability encountered in this disease. Of note, 1 subject in each cohort (3/21 subjects, 14.3%) experienced an increase of more than 100% in eosinophil levels at week 28 compared with eosinophil levels at week 8, but only subject 23, who had glucocorticoid therapy gradually reduced by 50% after the second mepolizumab infusion, had eosinophil levels substantially higher at week 28 compared with levels at weeks 0 through 8 (4710 vs 920 cells/mm³ [range, 50–1410 cells/mm³], respectively). All other subjects displayed eosinophil levels at week 28 in the range of levels measured before therapy (weeks 0–8).

Studying mAbs in human subjects is generally limited because no control antibody is used. Other limitations relating to human studies are the heterogeneous characteristics of the study population and the range of severity of the disease state. Despite these limitations encountered in this study, it is impressive that the demonstrated responses were fairly consistent. We believe that IL-5 is an upstream pathway for eosinophilic disease because we have demonstrated that it is a key molecule in regulating the peripheral blood eosinophil level in a variety of eosinophilic disorders. Indeed, analysis of patients who started with eosinophil levels of greater than 1500 cells/mm³ revealed that mepolizumab decreased eosinophil levels from 2566 ± 1305 cells/mm³ ($31\% \pm 10\%$) to 50 ± 29 cells/mm³ ($1\% \pm 0\%$), a 52-fold decrease ($P=.03$, $n=6$). The ability of anti-IL-5 to control clinical parameters (which might be more heterogeneous) was not tested in this study. Rather, we have found conserved immunologic and hematologic effects.

In summary, we were able to (1) elucidate the hematologic and immunologic properties of humanized anti-IL-5 therapy in patients with diverse eosinophilic disorders; (2) demonstrate the ability of this therapy to dramatically induce sustained reductions in blood eosinophilia and the level of CCR3⁺ cells (as a marker of eosinophils by means of flow cytometry, with no changes in the mean number of CCR3 receptors per individual cell); (3) demonstrate that anti-IL-5 therapy *in vivo* reduces eosinophil activation *ex vivo*, providing supportive evidence for drug efficacy; and (4) define the existence of an endogenous IL-5 autoregulatory loop that has implications for anti-IL-5 dosing strategies. The identification of an IL-5/immunoglobulin complex after anti-IL-5 therapy defines the importance of further elucidating the structure and significance of this complex in patients. Given that mepolizumab has recently been demonstrated to offer steroid-sparing effects in patients with HES²¹ and is promising for the treatment of eosinophilic disorders,²⁰ this report provides timely novel translational information.

Clinical implications

Anti-IL-5 (mepolizumab) decreases eosinophil activation and numbers, regardless of baseline blood IL-5 levels. Increased levels of immunoreactive blood IL-5 levels observed after therapy likely reflect neutralized IL-5/mepolizumab complexes rather than active IL-5. Monitoring eosinophil levels after therapy is important.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

APC, Allophycocyanin; EE, Eosinophilic esophagitis; EGID, Eosinophilic-associated gastrointestinal disorders; *FIP1L1-PDGFR*, *FIP1L1*-platelet-derived growth factor receptor α fusion gene; FITC, Fluorescein isothiocyanate; HES, Hypereosinophilic syndrome; IL-5R, IL-5 receptor; mcf, Mean channel of fluorescence; PE, Phycoerythrin; PerCP, Peridinin chlorophyll-protein.

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Fig 1.

Effect of anti-IL-5 on circulating leukocytes and peripheral blood eosinophil counts. Study protocol (A) showing eosinophil responses represented by individual lines (B) and in paired individual changes before (week 8) and after (week 20) anti-IL-5 therapy (C). The percentage of eosinophils and lymphocytes (mean $\pm$ SD, n = 25) and the numbers of total white blood cell (WBC) counts expressed as $\times 10^3$ cells (k) per cubic millimeter are shown (D). A dot diagram of peripheral blood eosinophil levels is shown (E).

Fig 2.

Plasma IL-5 levels before and after anti-IL-5 therapy. Plasma IL-5 levels represented for patients and healthy control subjects (NL; **A**) with paired individual changes (**B**) before (week 8) and after (week 20) anti-IL-5 therapy are shown. Dots under detection limit (*dashed line*) are extrapolated data in a log $\times$ log scale curve analysis and do not represent true values. The *horizontal bars* represent mean values.

Fig 3.

Molecular analysis of plasma IL-5. The average of subject and control samples are presented as the original IL-5 level detected at week 20 and after filtration in the upper and lower chambers (see the Methods section for details). Recombinant IL-5 at 100 pg/mL was used as a control to demonstrate that IL-5 is normally found in the lower chamber (A). The upper chamber fraction precipitation with nonsaturating quantities of protein A/G (50 μL) in representative samples and a control sample of T-cell supernatant after PHA stimulation is shown (B). A representative protein A/G dose-response curve is shown (C). The combined results of subjects' plasma (week 20) samples before and after protein A/G precipitation with saturating amounts (200 μL) is shown (D). The dashed line represents the detection limit value. Horizontal bars represent mean (SD) values (n = 12). ** $P < .01$, *** $P < .001$.

Fig 4.

Effect of anti-IL-5 on peripheral blood CCR3⁺ cell levels and the expression of IL-5R α mcf in peripheral blood eosinophils. The percentage of CCR3⁺ cells in peripheral blood leukocytes (A and B) and the expression of IL-5R α mcf (C and D) are represented as group distribution (Fig 4, A and C) and paired analysis (Fig 4, B and D) for all study subjects before (week 8) and after (week 20) anti-IL-5 therapy. The *horizontal bars* represent mean values.

Fig 5.

Effect of anti-IL-5 on intracellular IL-5 production by T cells. Anti-IL-5 therapy increased the percentage of peripheral blood CD4⁺ and CD8⁺ cells producing intracellular IL-5. The percentage of CD4⁺ (A and B) and CD8⁺ (C and D) cells producing intracellular IL-5 is shown before (week 8) and after (week 20) anti-IL-5 therapy for all study subjects. The unpaired (Fig 5, A and C) and paired (Fig 5, B and D) data are shown. The horizontal bars represent mean values.

Fig 6.

Effect of anti-IL-5 therapy on peripheral blood eosinophil activation in response to eotaxins. Peripheral blood eosinophil activation was assessed based on eotaxin-induced eosinophil shape change *ex vivo* in response to eotaxin-1 (**A**), eotaxin-2 (**B**), and eotaxin-3 (**C**) in patients before (week 8) and after (week 20) anti-IL-5 therapy. Eosinophil shape changes in response to eotaxin-1 and eotaxin-2 are shown in a paired analysis; eosinophil shape changes in response to eotaxin-3 are shown in a nonpaired analysis. * $P < .05$, ** $P < .01$.

Table 1

Patient characteristics

Patient no.	Sex	Age (y)	Diagnosis	Systemic organ involvement*	F/P†	Atopy	Years of disease	Medications			Adverse events to therapy‡	Plasma IL-5 before therapy (pg/mL)			Eosinophils (cell/mm³)			Percentage of eosinophil recovery§
								previous	week 8	week 20		week 8	week 20	week 28	week 8	week 20	week 28	
Cohort A																		
1	F	48	HES	C, D, P, H, N, G	–	N	11	P, PPI, IFN-α HU, MTX	MP 8 + MTX 17.5	NC	–	2	198	95	105	53		
2	M	55	HES	C, D, P, H	–	Y	2	P, PPI, HU, inh st	P 10 + HU 1000	NC	–	1	1500	58	19	1		
3	F	40	HES	C, D	–	Y	4	P	P 5 + inh st	NC	–	3	630	71	170	27		
4	M	18	EE		–	Y	10	P, Crom, iv steroids, sw FP	None	NC	Headache	7	402	82	50	12		
5	F	53	HES	C, D, P, H	–	Y	2	HC, IFN-α	HC 5	NC	–	2	1258	59	114	9		
6	F	44	HES + EGID	D, H, N, G	–	N	4	P, HU, IFN-α imatinib	None	NC	Chills	15	4620	72	ND	ND		
7	M	46	HES + EGID	D, P, G	–	Y	26	Bud, Crom, P, PPI	Bud 9	NC	–	3	612	264	1337	218		
8	F	41	EE		–	Y	34	P, 6-MP, Crom, diet, sw FP	Diet only	NC	Headache, hypotension	2	840	60	57	7		
9	M	22	EE		–	Y	11	Inh st	None	NC	–	4	280	57	57	20		
10	F	21	EE		–	Y	9	P, sw FP, Diet, PPI	PPI + diet	NC	–	3	240	79	60	25		
11	M	20	EE		–	Y	9	Elemental diet, PPI	PPI	NC	Headache, burning eyes sensation	2	490	50	58	12		
12	F	56	HES	D, N, G	–	Y	4	P, alprazolam	P 5	Trial withdrawal	–	0	10370	9070§	NA	NA		
13	F	19	HES + EGID	G	–	Y	3	Becl, diet, inh st, imatinib	Becl 6	Trial withdrawal	Flushing, abdominal colic pain, dyspnea, metallic taste	24	320	82§	NA	NA		
14	F	40	EE		–	N	10	sw FP, PPI	PPI	NC	–	2	260	50	20	8		
Cohort B																		
15	M	48	HES + EE	D, H, G	–	N	2	P, PPI	P 25/20 Alt. d	P 17.5/17.5 Alt. d	Headache, metallic taste	3	1125	120	1440	128		
16	M	53	HES	C, P, H, N	+	N	3	P, HU, imatinib	P 5 + HU 1000	P 5 + HU 750	–	4	1650	32	147	9		
17	F	38	HES	D, P, H, G	–	Y	12	P, Om 375 (anti-IgE)	P 15, Om 375, Inh st, PPI	P 11.25 and same	–	2	360	0	10	3		
18	M	40	HES + EGID	D, P, H, G	–	Y	30	P, PPI, diet, inh st, Crom	P 7.5	P 5.5, inh st, H2	–	30	1830	75	1200	66		
19	F	33	HES/Churg Strauss + EGID	C, P, N, G	–	Y	10	P, MTX, CTX, mofetil, imatinib	P 10, CTX 2	P 7.5, CTX same	–	4	320	0	ND	ND		
20	M	47	EGID		–	Y	10	Bud, sw FP, ML, H2	Bud 9, sw FP, ML and H2	Bud 6 and same	–	3	150	40	43	29		
Cohort C																		
21	M	39	EP		–	Y	6	P, inh st	P 12.5 e.o.d.	P 6 mg e.o.d.	–	1	120	100	100	83		
22	F	39	EP		–	Y	4	P, inh st	P 15	P 7.5	–	0	200	0	120	60		
23	M	19	HES	D, P, H, G	–	N	3	P, PPI, alendronate sodium	P 17	P 10	–	4	1430	40	4710	329		
24	M	57	HES	C, D, H	–	Y	3	P	P 10	P 5	–	4	3780	60	600	16¶		

Patient no.	Sex	Age (y)	Diagnosis	Systemic organ involvement [*]	E/P [†]	Atopy	Years of disease	Medications				Eosinophils (cell/mm ³)				Plasma IL-5 before therapy (pg/mL)	Adverse events to therapy [‡]			Percentage of eosinophil recovery [§]
								previous	week 8	P 10	carvedilol	week 20	Week 8	Week 20	Week 28	Week 57				
25	M	37	HES	C, P, N, G	–	Y	2	P, inh st, lisinopril, carvedilol	P 10			P 6		2016	0	57	–		ND	3

Glucocorticoid doses are in milligrams per day.

P, Prednisone; PPI, proton pump inhibitor; HU, hydroxyurea (mg/d); MTX, methotrexate (mg/wk); MP, methylprednisolone; NC, no change; Inh st, inhaled steroids; EE, eosinophilic esophagitis; Crom, oral cromolyn; sw FP, swallowed fluticasone propionate; HC, hydrocortisone; ND, not determined; Bud, budesonide; 6-MP, 6-mercaptopurine; NA, not applicable; Bezlo, beclomethasone; Alt. d, alternating days; Om, omalizumab (anti-IgE, mg every 2 weeks); CTX, cyclophosphamide (g/mo); ML, montelukast; H2, antihistamines; EP, eosinophilic pneumonia; e.o.d, every other day.

^{*} Systemic organ involved: C, cardiac; D, dermatologic; P, pulmonary; H, hematologic; N, neurologic; G, gastrointestinal.

[†] FIP1L1-PDGFRA fusion gene.

[‡] Adverse events listed are those possibly attributed to drug.

[§] (Week 12) only 2 infusions of mepolizumab.

^{||} Percentage of the increase in eosinophil levels at week 28 compared with week 8 (before treatment).

[¶] Patient increased his prednisone dose to 20 mg at week 28; he was taking 5 mg at week 24, and eosinophil levels were 1600 cells/mm³ (recovery of 42%).