Brief Definitive Report

INTERLEUKIN 5 AND PHENOTYPICALLY ALTERED EOSINOPHILS IN THE BLOOD OF PATIENTS WITH THE IDIOPATHIC HYPEREOSINOPHILIC SYNDROME

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The idiopathic hypereosinophilic syndrome (IHES) is characterized by sustained peripheral blood eosinophilia associated with organ involvement in the absence of a defined etiology (1). The isolation of eosinophils from patients with IHES has resulted in the identification of a population of eosinophils of a lower sedimentation density (hypodense) than that of eosinophils purified from healthy individuals (normodense) (2, 3). As compared with normodense eosinophils, hypodense eosinophils exhibit increases in calcium ionophore A23187-stimulated leukotriene C_4 (LTC_4) generation (4) and antibody-dependent helminthic cytotoxicity (3). The percentage of circulating hypodense eosinophils directly correlates with the degree of peripheral blood eosinophilia (5), but the factors that may regulate the eosinophil phenotype in IHES have not been determined previously.

A continuous exposure of normodense eosinophils to recombinant human granulocyte/macrophage colony-stimulating factor (rGM-CSF) (6), human rIL-3 (7), purified murine IL-5 or human rIL-5 (8) allows them to be maintained ex vivo for at least 2 wk. During this interval, the eosinophils become hypodense and exhibit both augmented calcium ionophore–stimulated LTC_4 generation and antibody-dependent cytotoxicity against *Schistosoma mansoni* larvae. The similarity between hypodense eosinophils, which are generated in vitro by the exposure of normodense eosinophils to specific cytokines, and hypodense eosinophils, which are freshly isolated from the peripheral blood of patients with IHES, prompted an analysis of eosinophil phenotypes and cytokine activities in the peripheral blood of patients with IHES.

Materials and Methods

Patients. Three patients underwent diagnostic studies to rule out collagen-vascular diseases, helminthic infections, neoplasia, drug reactions, atopy, or asthma, and each fulfilled

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the diagnostic criteria for IHES (1). Patient 1 was a 28-yr-old male with a 4-yr history of eosinophilia and hepatosplenomegaly complicated by pulmonary infiltrates, hypoxemia, and an acute peripheral myopathy. Patient 2 was a 29-yr-old male with a 3-yr history of eosinophilia complicated by endomyocardial fibrosis and thrombosis resulting in mitral regurgitation. Patient 3 was a 62-yr-old male with a 6-yr history of eosinophilia complicated by interstitial nephritis, cortical blindness, and seizures. None of these patients responded clinically to optimal doses of corticosteroids. Patient 1 was evaluated while receiving no therapy, or therapy with hydroxyurea, corticosteroids, cyclosporin A, or combinations thereof. Patient 2 was evaluated while being treated with leukoplasmapheresis and vincristine. Patient 3 underwent study while receiving cyclophosphamide and corticosteroids.

Eosinophil Isolation. Eosinophils were isolated from the peripheral blood of 21 different reference donors, who were healthy or were diagnosed with allergic rhinitis and/or asthma. Eosinophils were purified from the peripheral blood of the three patients with IHES and the reference donors by the centrifugation of individual dextran (BDH Chemicals, Poole, England) sedimented erythrocyte/leukocyte preparations through discontinuous layers of metrizamide (Nyegaard and Co., Oslo, Norway) of 18–24% (wt/vol) (6). Eosinophils recovered from the 22/23 and 23/24% metrizamide interfaces and the cell pellet were designated as normodense. Eosinophils recovered from the medium/18, 18/20, and 20/21% metrizamide interfaces were designated as hypodense (6). Initial cell viability in all experiments was >98% as assessed by Trypan blue (Gibco Laboratories, Grand Island, NY) exclusion.

Eosinophil Viability in Culture. Freshly isolated eosinophils ($2–10 \times 10^5$ cells) were suspended in 2 ml of enriched medium (RPMI 1640 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml of gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids and 10% [vol/vol] FCS serum [Gibco Laboratories]), and were maintained in 35-mm plastic culture dishes at 37°C in a 5% CO_2 atmosphere under various conditions (6): enriched medium alone; enriched medium supplemented with 10 pM rGM-CSF (expressed in monkey COS cells and provided by Dr. J. Gasson, University of California at Los Angeles, CA) in the presence of a monolayer of mouse 3T3 fibroblasts; and enriched medium supplemented with 25% (vol/vol) serum from Patient 1 with IHES. Neutrophils and mononuclear cells did not survive beyond 48 h of culture.

For experiments in 96-well, flat-bottomed microtiter plates, freshly isolated eosinophils ($5–10 \times 10^4$ cells) were suspended in 200-μl of enriched medium alone or medium supplemented with 10 pM rGM-CSF, 10 pM rIL-3 (expressed in COS cells and generously provided by Dr. Y.-C. Yang, Genetics Institute, Cambridge, MA), 1 pM IL-5 (purified from the conditioned medium of the helper T cell line, D10.G4.1, and generously provided by Dr. D. T. McKenzie, University of California at San Diego, La Jolla, CA), 1 pM rIL-5 (expressed in COS cells and provided by Dr. S. C. Clark, Genetics Institute), or defined concentrations of human serum or plasma (7). For some experiments, various dilutions of heat-inactivated (56°C for 30 min) neutralizing rabbit polyclonal antiserum against rGM-CSF (provided by Dr. J. Gasson) or rIL-3 (provided by Dr. Y.-C. Yang), or a rat mAb against murine IL-5 (9) (provided as unpurified ascites fluid by Dr. K. Takatsu, Kumamoto University Medical School, Honjo, Kumamoto, Japan) were preincubated for 150 min at 37°C with enriched medium containing heat-inactivated FCS with or without added cytokine or human serum or plasma. The effect of these treatments on cell viability was determined by Trypan blue exclusion after 48–72 h of culture.

Eosinophil Functional Studies and Density Gradient Sedimentation. Freshly isolated eosinophils were assessed for their ability to kill antibody-coated and uncoated *S. mansoni* larvae after 24 h (6). The calcium ionophore–stimulated generation of LTC_4 by eosinophils before and after 7 d of culture was assessed by stimulating eosinophils with 2.5 μM A23187 (Calbiochem-Behring Corp., San Diego, CA), and the methanolic extract was analyzed by RIA (10).

Results

Comparative Functional Studies of Freshly Isolated Hypodense and Normodense Eosinophils. Less than 3% of the eosinophils from the reference donors were hypodense. The normodense eosinophil gradient fractions from the reference donors contained 90

± 1% eosinophils and 10 ± 1% neutrophils (mean ± SEM; n = 7). 61 ± 23% (n = 3; $p < 0.05$) of the eosinophils isolated from the three patients with IHES were hypodense; these fractions (18/20 and 20/21% metrizamide) contained 78 ± 6% eosinophils, 17 ± 4% neutrophils, and 5 ± 3% mononuclear cells. The normodense eosinophil gradient fractions from the patients with IHES contained 98 ± 1% eosinophils and 2 ± 1% neutrophils.

In the absence of immune sera, eosinophils from the reference donors killed <5% of the larvae (n = 3), normodense eosinophils from the patients killed 8 ± 7% (n = 2) of the larvae, and hypodense eosinophils from the patients mediated a striking helminthic cytotoxicity of 30 ± 8% (n = 3; $p < 0.05$). With antisera, cytotoxicity mediated by the eosinophils from the reference donors was increased to 14 ± 2% (n = 17), and normodense and hypodense eosinophil populations from the patients with IHES increased their antibody-dependent helminthic cytoxicity to 40 ± 10 and 50 ± 17%, respectively (n = 3; $p < 0.001$). In comparison to the 20 ± 9 ng of $LTC_4/10^6$ cells generated by the calcium ionophore-stimulated normodense eosinophils from the reference donors (n = 15), both normodense and hypodense eosinophil populations from the patients produced strikingly augmented quantities of LTC_4 of 69 ± 12 and 98 ± 9 ng/10^6 cells, respectively (n = 3; $p < 0.001$).

Comparative Ex Vivo Viability of Freshly Isolated Normodense and Hypodense Eosinophils. Only 17 ± 8% of the initial number of eosinophils from the reference donors were still viable after 48 h of culture. Greater survival was manifest by the normodense eosinophils isolated from the patients with IHES (33 ± 8% at 48 h). The percentage of hypodense eosinophils from the patients that survived in culture after 48 h in the absence of exogenous cytokines was 73 ± 7% (Fig. 1). As assessed by light microscopy, no mitotic figures were observed in any population of cultured eosinophils. After 7 d, rGMCSF-mediated survival in culture with 3T3 fibroblasts was 51 ± 7% (n = 3), 52 ± 20% (n = 2), and 66 ± 10% (n = 3) for normodense eosinophils from the reference donors, normodense eosinophils from the patients with IHES, and hypodense eosinophils from the patients with IHES, respectively.

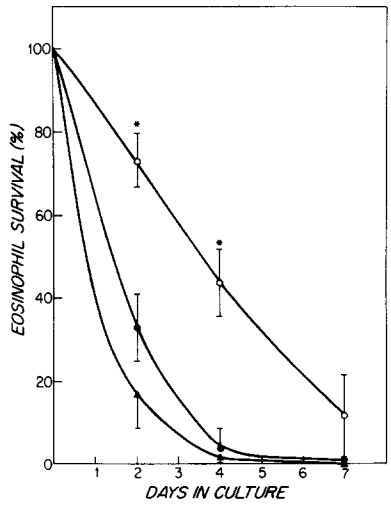

FIGURE 1. Time course for the survival of freshly isolated eosinophil populations. Hypodense (○) and normodense (●) eosinophils from the patients with IHES and normodense (▲) eosinophils from the reference donors were maintained in enriched medium alone. Data for hypodense eosinophils from the patients with IHES (n = 3) and normodense eosinophils from the reference donors (n = 5) are expressed as the mean ± SEM. Data for normodense eosinophils from the patients with IHES (n = 2) are expressed as the mean and the span of the range. Inadequate numbers of normodense eosinophils were obtained from Patient 2 for evaluaton. Statistical comparisons for eosinophil populations from patients with IHES were made to normodense eosinophils from the reference donors (*, $p < 0.05$).

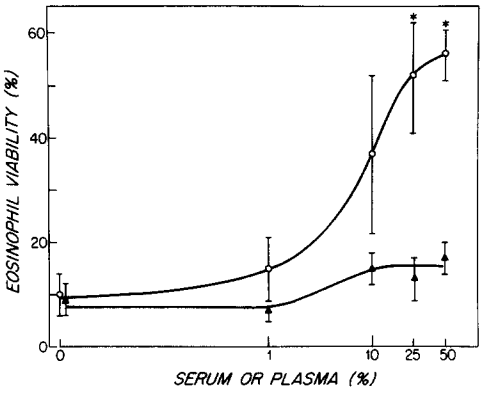

FIGURE 2. Influence of sera or plasma on eosinophil viability ex vivo. Normodense eosinophils from the reference donors were cultured for 48–72 h in enriched medium supplemented with incremental concentrations of individual serum (Patients 1 and 3) or plasma (Patient 2) samples from the patients with IHES (○) (n = 3), or serum or plasma from the individual reference donors (▲) (n = 5). Data are expressed as the mean ± SEM. For eosinophils maintained in enriched medium supplemented with sera or plasma from the IHES patients, statistical comparisons were made to replicate eosinophils maintained in medium supplemented with sera or plasma from the reference donors (*, $p < 0.05$).

Effect of Serum and Plasma from Patients with IHES on the Viability of Normodense Eosinophils. When normodense eosinophils from the reference donors were cultured for 48–72 h in enriched medium supplemented with serum or plasma from individual patients with IHES, a dose-dependent enhancement of eosinophil viability occurred (Fig. 2). When dose-response curves for rGM-CSF and serum from Patient 1 were performed in parallel on replicate normodense eosinophils (n = 4), 5 pM rGM-CSF sustained eosinophil viability at the same level as 10% serum.

Normodense eosinophils from two different reference donors were cultured for 7 d in enriched medium supplemented with 25% serum from Patient 1. Eosinophil survival was 66 ± 2%, and 77 ± 1% of these cells were converted to hypodense sedimenting cells. These cultured eosinophils generated 145 ± 16 ng $LTC_4/10^6$ calcium ionophore-stimulated cells (n = 2), as compared with 35 ± 1 ng from freshly isolated normodense eosinophils from the same donors.

Antibody Neutralization of the Viability Sustaining Activity in IHES Serum and Plasma. At optimal dilutions of 1:250 (vol/vol) for anti-GM-CSF and anti-IL-3, and 1:100 for anti-IL-5 as defined by inhibition of the appropriate cytokine, no crossreactivity in neutralizing activities was noted. Anti-IL-5 neutralized the viability-sustaining activity of 1 pM rIL-5 in a dose-dependent manner with complete neutralization at dilutions ≤1:1,000. Preincubation of 25 or 10% sera from Patients 1 and 3 and plasma from Patient 2 with a 1:100 dilution of anti-IL-5 diminished mean eosinophil viability after 72 h from 58 ± 5 and 44 ± 12% to 26 ± 3 and 20 ± 1%, respectively (Fig. 3). 1:250 dilutions of anti-GM-CSF and anti-IL-3 did not attenuate the viability of normodense eosinophils maintained 72 h in enriched medium supplemented

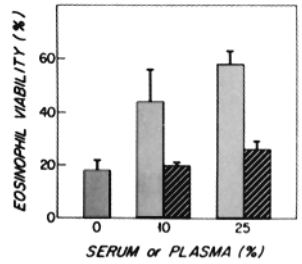

FIGURE 3. Influence of neutralizing antibody against IL-5 on the viability-sustaining activity of IHES sera or plasma. Normodense eosinophils from the reference donors were cultured for 48–72 h in enriched medium alone, or medium supplemented with 10 or 25% serum or plasma from the three individual IHES patients (*stippled bars*). Replicate serum or plasma samples were preincubated with 1:100 dilutions of neutralizing antibody against IL-5 (*crosshatched bars*). Data are expressed as the mean ± SEM (n = 3).

with 25 or 10% serum from Patients 1 and 3. Plasma from Patient 2 was not evaluated with these antisera. The viability for replicate normodense eosinophils maintained in enriched medium alone was 18 ± 4% (n = 3). The addition of anti-IL-5 to serum containing rGM-CSF during the preincubation period did not alter the increase in viability after 72 h which was 60 ± 3% as compared with 21 ± 8% without GM-CSF (n = 2). Replicate eosinophils maintained in medium alone, medium supplemented with 10 pM rGM-CSF alone, or medium supplemented with 25% serum alone had a viability of 15 ± 5%, 77 ± 2%, and 64 ± 14% (n = 2), respectively.

Discussion

The similar properties between the in vivo-derived hypodense sedimenting eosinophils associated with a variety of eosinophilic disorders, and the hypodense eosinophils generated in vitro by the exposure of normodense peripheral blood-derived eosinophils to GM-CSF, IL-3, or IL-5, prompted a detailed analysis of the eosinophil phenotypes and cytokine activities in the peripheral blood of three patients with corticosteroid-unresponsive IHES. 61% of the eosinophils from the patients with IHES were hypodense. Both hypodense and normodense eosinophil populations from the IHES patients demonstrated an augmented capacity to generate LTC_4 upon calcium ionophore stimulation (5-fold and 3.5-fold, respectively) and an enhanced capacity to mediate antibody-dependent cytotoxicity against *S. mansoni* larvae (3.5-fold and 3-fold, respectively), as compared with eosinophils from the reference donors. Hypodense eosinophils from the patients were able to mediate significant cytotoxicity against the larvae even in the absence of antibody sensitization. The ability to kill unopsonized *S. mansoni* larvae may be relevant to the finding of eosinophil degranulation in various tissues of patients with IHES (11). The hypodense eosinophils exhibited a $t_{1/2}$ for ex vivo viability of 3.5 d, whereas <10% of the normodense eosinophils from either the reference donors or the patients survived an equivalent duration. These in vitro findings may be relevant to in vivo circumstances, since a previous study which made no distinction for eosinophil density indicated that autologous chromium-labeled peripheral blood eosinophils from patients with IHES may have a prolonged life span in vivo (12).

Serum or plasma from the patients with IHES contained an activity that in a dose-dependent manner was capable of conferring extended viability, augmented function, and the property of hypodensity to normodense eosinophils from the reference donors. An ED_{50} of 8% serum or plasma was extrapolated for the maintenance of eosinophil viability ex vivo, and this concentration was equivalent in activity to ~5 pM GM-CSF. No similar activity was detected in the serum and plasma of the reference donors. A monospecific neutralizing antibody against IL-5 completely abolished this activity, but antisera against GM-CSF or IL-3 were without effect.

The finding of abnormal quantities of immunoreactive IL-5 in the blood of patients with IHES could account for several clinical aspects of this disorder. IL-5 is unique among the hematopoietins in its ability to cause granulocytosis with a selective eosinophilia in vitro and in vivo (13). These altered eosinophil phenotypes with a putative pathobiologic action in patients with IHES may arise as a consequence of abnormal quantities of IL-5 in the peripheral blood.

Summary

We report that the hypodense eosinophil population in three patients with corticosteroid-unresponsive IHES was uniquely long lived ex vivo in the absence of exogenous cytokines. Serum or plasma from these patients conferred prolonged viability ex vivo to normodense eosinophils from reference donors and converted them to a functionally activated hypodense phenotype. In that antibody against IL-5 neutralized this activity in IHES serum, excessive quantities of this cytokine may account for the characteristic eosinophilia and long-lived, functionally augmented eosinophil phenotype in this disorder.

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