Ultrastructural Study of Eosinophils From Patients With the Hypereosinophilic Syndrome: A Morphological Basis of Hypodense Eosinophils

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We investigated the ultrastructural characteristics and the granule major basic protein (MBP) content of hypodense eosinophils from patients with the hypereosinophilic syndrome who had at least 90% hypodense eosinophils in their peripheral blood and compared these cells to normodense eosinophils from normal persons. The hypodense cells (density < 1.082) contained significantly less MBP than normodense (density > 1.082) eosinophils ($P < .001$) as measured by radioimmunoassay (RIA). Electron microscopic examination demonstrated a mean of 25.0 ± 4.4 ($\bar{X}$ ± 1 SD) granules per hypodense cell, compared to 30.6 ± 8.4 granules per cell in the normodense group ($P < .1$). The most striking difference between the hypodense and normodense eosinophils was the small individual granule size ($\bar{X}$ = .14 ± .05 v .26 ± .05 micron^2, respectively, $P < .001$), and the smaller total granule area (3.2 ± 1.8 vs 7.7 ± 3.1 μm^2, respectively, $P < .001$). Because the cytoplasmic areas were similar in the two groups, the mean percent area of cytoplasm occupied by granules was significantly lower in the hypodense group ($P < .001$). The finding of consistently smaller granules in the presence of equal or fewer granules per cell in the hypodense eosinophils may explain the lower MBP content and thus provide a morphologic basis for the low density of eosinophils in patients with the hypereosinophilic syndrome.

ALTHOUGH the eosinophil has the greatest buoyant density of human peripheral blood leukocytes, studies using density gradient centrifugation have shown a subpopulation with lighter density.[1-5] Such eosinophils have been referred to as "hypodense." Eosinophils from patients with peripheral blood eosinophilia due to a spectrum of underlying causes, including allergic, immunologic and parasitic diseases, appear to be different from those of normal individuals with respect to a variey of parameters and are composed of varying populations of low-density cells.[1-6] Indeed, many of the abnormalities observed in eosinophils from patients with eosinophilia have been associated specifically with the hypodense subset, including decrease in cellular eosinophilic cationic protein (ECP) content, increase in spontaneous O_2 consumption, increase in surface receptors for immunoglobulin and complement, increased lactic dehydrogenase (LDH) isoenzyme 5, and enhanced cytotoxicity.[2-5,7,8] While such functional differences between hypodense and normodense eosinophils have been described, there have been few studies of the structural characteristics of low-density eosinophils.

In a previous study we defined hypodense eosinophils as cells with densities less than 1.082 g/mL in the Percoll gradients, based on comparison with the density distribution profiles of eosinophils from normal individuals.[9] More than 90% of the peripheral blood eosinophils from normal subjects had a density greater than 1.082, whereas in patients with the hypereosinophilic syndrome (HES) more than 90% of the peripheral blood eosinophils were hypodense. These differences suggested that study of the ultrastructure of hypodense as compared with normodense eosinophils could be performed in patients with HES without need to isolate the hypodense cells. Here we investigated the ultrastructural morphology and major basic protein (MBP) content of hypodense eosinophils using peripheral blood cells from patients with HES or other underlying disease who had greater than 90% hypodense eosinophils to determine the morphological basis of the low density of these eosinophils.

MATERIALS AND METHODS

Subjects. Peripheral blood eosinophils from 20 patients with eosinophilia were initially studied for density distribution profiles by the method described below. Six of the 20 patients were chosen for the present study because they had marked peripheral blood eosinophilia and more than 90% of their eosinophils were hypodense. Four of these patients had HES, one had episodic angioedema,[10] and one had hepatitis (Table 1).

Density distribution analysis. Fractionation of eosinophils was performed by centrifugation of leukocytes on Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) density gradients.[9-11] Briefly, Percoll gradients consisting of 1.5 mL 1,100, 3 mL 1.090, 3 mL 1.085, and 3 mL 1.080 g/mL Percoll solution were prepared. Peripheral blood leukocytes were obtained by sedimenting erythrocytes in heparinized blood with 6% Dextran T70 (Pharmacia Fine Chemicals) and resuspending the supernatant leukocytes in Percoll solution of 1.070 g/mL density. Percoll gradients were overlaid with these leukocytes (1×10^8 cells/2 mL vol), centrifuged at 1,600 g, 10°C, for 20 minutes, and cells were harvested from the gradients in 1.0-mL fractions. One hundred-microliter samples were withdrawn from each fraction for determination of density. The remaining cells were washed twice, stained with Randolph's phloxine-methylene blue, and counted in a hemocytometer. Cytocentrifuged specimens were also prepared and stained with Wright's stain.

The relationship between refractive index (RI) and density was determined initially by weighing 10-mL volumes of Percoll solutions of differing density in pycnometers (Arthur H. Thomas Co, Philadelphia) and by measuring RI at 22°C with an ABBE-3L refractometer (Bausch and Lombe, Rochester, NY). The linear relationship between density and RI was determined to be: density = (RI-1.18546)/0.14821 (R^2 = .9997). Thereafter density was determined by measuring the RI and converting the RI values to density using the equation for the least-squares regression line.

Density distribution profiles of eosinophils were obtained by expressing eosinophil recovery in each fraction as a percentage of

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Table 1. Hypodense Eosinophils

Patient	Disease	Eos %	Eos/ μL	% Hypodense Eos	Peak Density	MBP/10^6 cells (ng)	MBP Plasma ng/mL
1*	HES†	68	18,630	99.6	1.076	4,100	5,259
2*	HES	50	9,050	99.7	1.075	3,502	2,339
3*	HES	69	6,624	97.9	1.075	3,257	2,248
4	HES	57	2,793	96.0	1.077	3,819	1,867
5	Episodic Angioedema	69	13,041	90.0	1.076	3,197	2,408
6	Hepatitis	21	1,156	92.8	1.076	3,538	1,420
	Mean =	55.7	8,549	96.0	1.076	3,569	2,590
	SD =	18.5	6,535	3.9	0.001	342	1,359

*Eosinophils from these subjects studied by electron microscopy.

†HES = hypereosinophilic syndrome. Patient 1 subsequently died due to the HES, patient 2 recovered with treatment and now does not require treatment, and patient 3 is controlled on prednisone and hydroxyurea.

total eosinophils recovered from the gradients and by plotting these percentages against the density of the fractions. The recovery of eosinophils from gradients was 92% ± 3% for the normal subjects and 95% ± 2% for the hypodense group.

Preparation of eosinophil extracts and plasma. Eosinophil-enriched fractions were selected, and the eosinophil concentration in each fraction was adjusted to 1×10^6 cells/mL. Cells were lysed with 0.5% Nonidet P-40 (Sigma Chemical Co, St. Louis), centrifuged at 400 g for ten minutes, and the resulting clear supernatants were frozen at −70°C. Plasma specimens were obtained from a portion of the blood samples used for the assessment of eosinophil density distribution and frozen at −20°C.

Measurement of MBP levels. MBP concentrations were measured in cell extracts and plasma by a double antibody radioimmunoassay (RIA), as previoulsy described.[12]

Electron microscopy. Peripheral blood, obtained at the same time as blood drawn for determination of density distribution, was centrifuged at 50 g for seven minutes. The buffy coat layer was aspirated through a long needle attached to a syringe and was immediately placed in 3% glutaraldehyde in .067 mol/L phosphate buffer, pH 7.2. After fixation for six hours the preparation was centrifuged, and the supernatant was removed and replaced with .1 mol/L cacodylate buffer, pH 7.4. Cells were rinsed in three changes of .1 mol/L phosphate buffer (pH 7.2), postfixed in 1% OsO_4 in .1 mol/L phosphate buffer, and rinsed in three changes of water over 15 minutes. After en bloc staining with 2% uranyl acetate for 15 minutes at 60°C, cells were rinsed in water and dehydrated through a series of increasing concentrations of ethanol. Cells were infiltrated for one hour with a 1:1 mixture of Spurr (Electron Microscopy Sciences, Fort Washington, PA) and ethanol, for one hour with 3:1 Spurr/ethanol, and overnight in pure Spurr. Cell pellets were transferred to BEEM capsules (Polysciences, Warrington, PA) and embedded in Spurr overnight at 70°C. Thin (600 A) sections were mounted on copper grids and examined with a Phillips 201 transmission electron microscope. Sections contained variable numbers of eosinophils randomly scattered among other leukocytes and erythrocytes. Selection of eosinophils for analysis was performed in a nonbiased manner as follows: starting at one corner of a thin section, each eosinophil was photographed at a constant magnification (excluding only cells without nuclei). At least 40 cells from each patient and a total of 43 cells from the two normal individuals were photographed.

Morphologic analyses. Using the electron photomicrographs obtained as described above, which were all photographed at the same magnification, the number of eosinophil granules, the number of granules showing core lucency, and the number of lipid bodies per cell were determined. Only granules containing cores were included in the counts. These analyses were obtained on a total of 174 eosinophils from three patients with HES and two normal individuals: 42 eosinophils from patient 1, 41 from patient 2, 48 from patient 3, and 43 from the two normal individuals. Because the patients' cells were not significantly different from those of the normal individuals on the basis of these parameters and because, by inspection, there appeared to be differences between the size of the granules from patient 3 compared to the granules of the normal individuals, we measured the cross-sectional areas of the eosinophils, their nuclei, and their individual granules. These areas were determined, again using the photomicrographs of individual eosinophils, by tracing the perimeters of the cells, the perimeters of the nuclei, and the perimeters of the individual granules within the cell. These measurements were then translated into two-dimensional areas (in cm^2) of the whole cell, the nucleus, and the individual granules using a Hewlett-Packard 9810A calculator with a program for digitizing areas. These measurements were obtained from photomicrographs of 45 eosinophils (ten cells from each HES patient and 15 from the two normal individuals) at final magnification 25,000 and were converted to μm^2 by the formula: $\mu m^2 = (cm^2 \times 10^8)/25{,}000$.[2] The cytoplasmic area was calculated as the difference between total cell area and nuclear area. These 45 cells represent the first ten consecutive cells photographed from each of the three HES patients, and the first 15 (seven or eight from each) of the normal controls.

Statistical analyses. Differences between normodense and hypodense study groups were tested by Student's t test, and the correlations were examined by least-squares regression analysis with a Hewlett-Packard 9845B computer (Hewlett-Packard, Cupertino, CA), using program numbers 09845-15130 and 09845-15110, respectively).

RESULTS

Density distribution profiles. Centrifugation and fractionation on Percoll yielded 13 fractions with densities ranging from 1.068 to 1.102 g/mL. Figure 1A shows representative profiles of eosinophils from two normal individuals. Density distribution analyses of ten normal individuals showed peaks at densities of 1.085 to 1.090 g/mL and inflection points or nadirs near 1.082 g/mL, below which only 10% of eosinophils were found.[9] Based upon these findings we divided eosinophils into two populations: normodense (>1.082 g/mL) and hypodense (<1.082 g/mL); Tables 1 and 2. The density distribution profiles of eosinophils from six patients with eosinophilia are shown in Fig 1B. These patients were selected for the present study because greater than 90% of their peripheral blood eosinophils were hypodense. The peak density of the patients' cells ranged

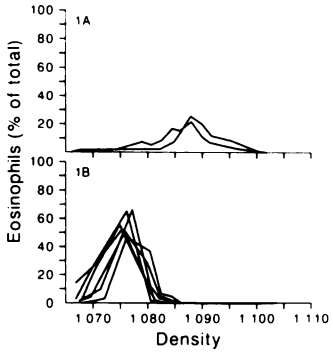

Fig 1. Buoyant density of eosinophils. Density distribution profile from two normal individuals (A) and six patients with peripheral blood eosinophilia (B) illustrates the significant difference ($P < .001$) in peak density between the two groups [$\bar{X}$ = 1.088 (A), $\bar{X}$ = 1.076 (B)].

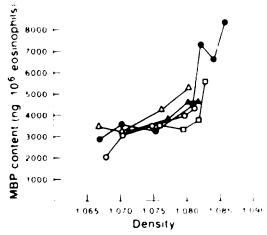

Fig 2. Eosinophil density and MBP content. The correlation between density and MBP cell content is illustrated by determinations of MBP content in cell fractions in five of six patients with eosinophilia. The MBP content of eosinophils increases as the buoyant density of the cell increases.

from 1.075 to 1.078 g/mL, with a mean of 1.076 g/mL; the percentage of hypodense eosinophils ranged from 90.0% to 99.7%, with a mean of 96% (Table 1).

MBP cell content and plasma levels. The MBP content of hypodense eosinophils was significantly lower than that of the eight normal individuals ($P < .001$), whereas the plasma MBP levels were markedly higher in the hypodense group ($P < .001$; Tables 1 and 2). In addition, there was an inverse relationship between cellular MBP content and plasma MBP level ($r = -0.72$, $P < .01$). Figure 2 shows that the MBP content of the cell fractions increases in concert with increasing density of the eosinophils. There was an inverse relationship between the degree of peripheral blood eosinophilia and cellular MBP content and between the percentage of hypodense eosinophils and the cellular MBP content (Table 3). Hence these results show that patients with eosinophilia have high plasma MBP levels and a high number of hypodense eosinophils that contain less MBP per cell than do eosinophils from normal individuals.

Electron microscopy. Table 4 shows that the number of granules and the number of lucent cores per cell did not differ between the three patients with HES and the two control subjects ($P < .1$ and $P < .2$, respectively). However, a higher percentage of granule cores showed lucency in the hypodense group ($P < .01$). Lipid bodies were infrequently seen, except in patient one. Inspection of Fig 3 shows that the

Table 2. Normodense Eosinophils

Control Subject	Eos %	Eos/ μL	% Hypodense Eos	Peak Density	MBP Content/ 10^6 cells (ng)	MBP Level in plasma (ng/mL)
1	1.7	121	6.0	1.089	10,935	351
2	4.6	118	9.0	1.088	7,332	258
3	1.7	87	18.2	1.088	ND	ND
4	2.6	129	18.2	1.088	ND	ND
5	1.3	80	6.6	1.089	7,321	326
6	2.5	108	4.2	1.089	9,638	308
7	1.3	97	22.8	1.086	8,470	282
8*	2.6	110	1.2	1.087	7,924	335
9*	2.5	115	9.6	1.087	10,143	342
10	2.0	153	7.4	1.089	7,189	254
Mean =	2.3	116	10.3	1.088	8,619	307
SD =	0.96	25	7.0	0.001	1,445	38

Abbreviations: ND, not determined.

*Eosinophils from these subjects were studied by electron microscopy.

Table 3. Correlations Among Eosinophils and Their Major Basic Protein Content

	% Hypodense Eosinophils n = 16*	MBP Content/ 10^6 Cells (ng) n = 14†	MBP Level in Plasma (ng/mL) n = 14†
Eosinophils/μL	$r = +0.747$	$r = -0.660$	$r = +0.947$
	$P < .001$	$P < .01$	$P < .001$
% Hypodense Eosinophils		$r = -0.916$	$r = +0.821$
		$P < .001$	$P < .001$
MBP Content/10^6 cells (ng)			$r = -0.717$
			$P < .01$

*n = 16 = ten normal control individuals plus six patients with eosinophilia.

†n = 14 = eight normal control individuals plus six patients with eosinophilia.

Table 4. Ultrastructural Features of Hypodense and Normodense Eosinophils*

Patient	No. Cells Analyzed	Hypodense Eosinophils: No. Granules/ Cell (Mean)	Cores with Lucency/ Cell (Mean): No.	Cores with Lucency/ Cell (Mean): %	No. Lipid Bodies/ Cell (Mean)
1	42	21.0	4.8	23.0	2.4
2	41	24.4	5.7	22.3	0.4
3	48	29.7	3.4	11.8	0.6
	Mean =	25.0	4.6	19.0	1.1
	SD =	4.4	1.2	6.3	1.1
		Normodense Eosinophils			
Control 8	15	36.5	5.9	16.0	0.5
Control 9	28	24.6	2.0	8.5	0.5
	Mean =	30.6	4.0	12.3	0.5
	SD =	8.4	2.8	5.3	0.0

*Counts based on individual photomicrographs of 174 cells at final magnification of 25,000 or 42,500.

granules of the HES patients, although approximately equal in number to those of a normal individual, appear smaller. Therefore this apparent difference was investigated in more detail.

Measurement of the area of cell organelles on photomicrographs showed that the total cellular area and the cytoplasmic area were not significantly different for hypodense as compared to normodense eosinophils ($P < .3$ and $P < .5$, respectively). The most striking difference between cells from the patients with eosinophilia and normal individuals was the size of individual granules. The granules were significantly smaller in the hypodense as compared to the normodense eosinophils ($\overline{X} = .14\ \mu m^2$ v $.26\ \mu m^2$, $P < .001$). Also, the total granule area per cell and percentage of cytoplasm occupied by granules were significantly lower in the hypodense group ($P < .001$; Table 5). The variations in granule size were not related to total cell area ($r = +.016$),

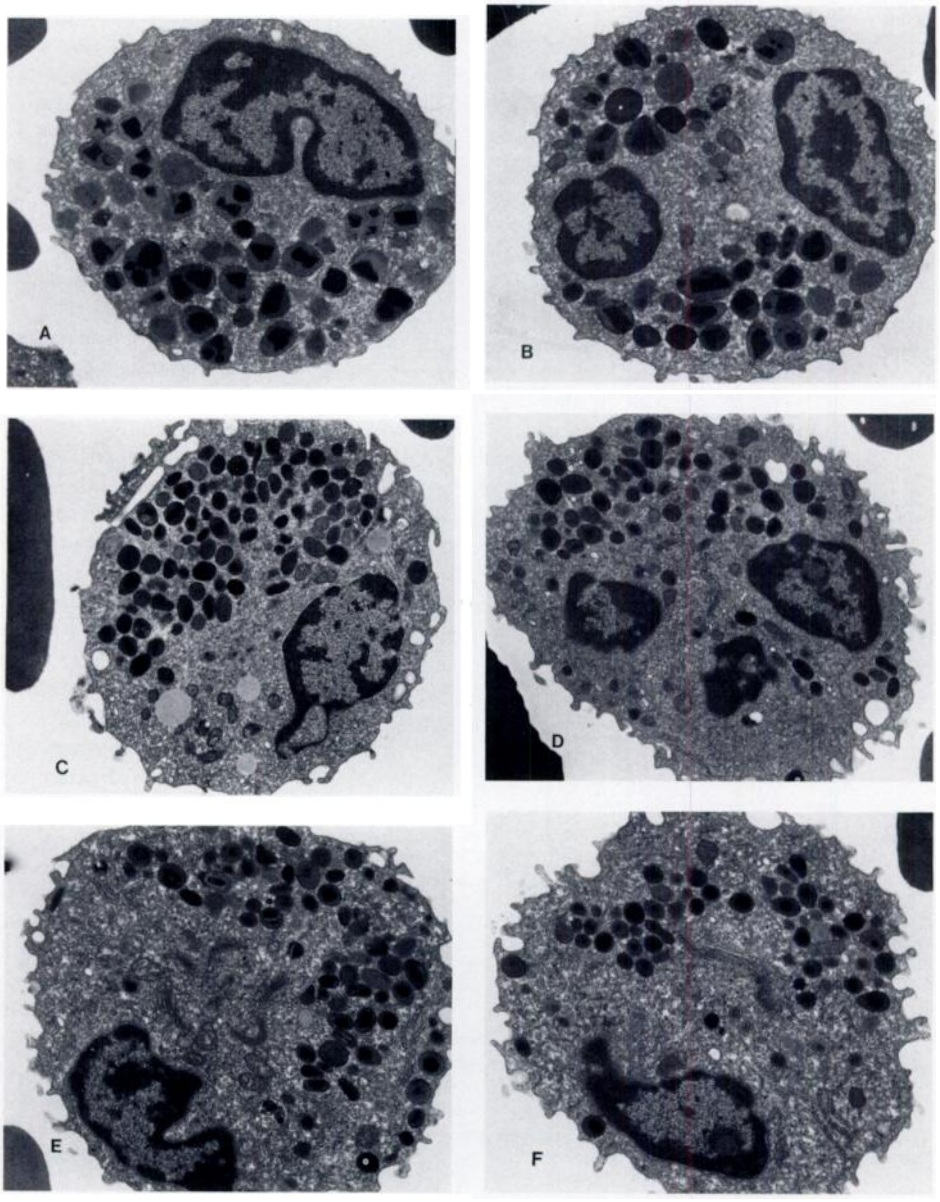

Fig 3. Comparison of the morphology of normodense and hypodense eosinophils. Normodense (A and B) and hypodense (C through F) eosinophils contain similar numbers of granules, but the individual granules are smaller in the cells from the HES patients. (Current magnification ×6,250).

Table 5. Ultrastructural Features of Hypodense and Normodense Eosinophils*

Patient	No. Cells Analyzed	No. Granules/ Cell (Mean)	Individual Granule Area μm^2 (Mean)	Total Granule Area/Cell, μm^2 (Mean)	Cytoplasmic Area/ Cell, μm^2 (Mean)	Cell Area μm^2 (Mean)	Total Granule Area X100/Cytoplasmic Area (Mean)
			Hypodense Eosinophils				
1	10	17.2	0.16	2.8	29.2	38.9	9.1
2	10	25.3	0.17	3.9	25.7	33.0	16.1
3	10	29.1	0.10	2.8	31.7	42.3	8.8
	Mean =	23.9	0.14	3.2	28.9	38.0	11.3
	SD =	9.9	0.05	1.8	7.5	9.2	6.5
			Normodense Eosinophils				
Control 8	7	41.8	0.23	9.8	34.7	41.5	28.0
Control 9	8	20.3	0.29	5.9	28.8	38.5	21.1
	Mean =	30.3	0.26	7.7	31.5	39.9	24.3
	SD =	13.5	0.05	3.1	6.1	7.0	7.7

*Measurements obtained in cm^2 and converted to μm^2 [$\mu m^2 = (cm^2 \times 10^8)/25{,}000^2$]; based on individual photomicrographs of 45 cells at final magnification of 25,000.

cytoplasmic area ($r = -.164$), or number of granules per cell ($r = +.094$).

DISCUSSION

In spite of the increasing evidence of functional differences between hypodense and normodense eosinophils, there has been a paucity of data concerning the morphologic differences between these two groups. Our patients with HES showed greater than 96% low-density eosinophils in their peripheral blood. Ultrastructural examination of light-density and normal-density eosinophils from these patients and from normal individuals showed that there was no significant difference between the number of granules per cell or the cell size, although there was a wide range in the number of granules per cell in the two normal subjects (Tables 4 and 5). Analyses of granule two-dimensional areas showed that hypodense eosinophils had significantly smaller individual granules and less total granule area per cell. The decreased ratio of total granule area to cytoplasmic area may explain the low density of the eosinophils from our patients with the HES. Because the crystalloid of the granule is composed of MBP,[13-15] one would expect that cells with less total granule area would have less MBP, and indeed the patients with the HES had significantly lower cellular MBP content than the normodense control cells (Fig 4). This finding, in addition to previous observations of decreased ECP content in hypodense eosinophils,[3] suggests that the decrease in protein content of the cells is an important factor in explaining the lower density of these eosinophils.

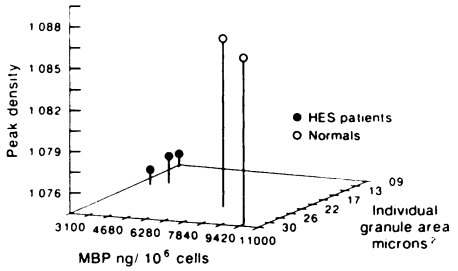

Fig 4. Relationship between eosinophil density, MBP cell content, and granule area (n = 5). There is a positive correlation between granule area and MBP cell content ($r = +0.946$, $P < .02$) and between granule area and peak density ($r = +0.889$, $P < .05$). Thus low-density eosinophils have smaller granules and less MBP.

Eosinophils from patients with peripheral blood eosinophilia have been reported to show cytoplasmic vacuoles and degranulation.[16-19] However, attempts at quantitating these findings have been described only at the light microscopic level, and detailed counts of granule numbers have not been reported. Spry and Tai[18] studied four patients with Loffler's cardiomyopathy and marked eosinophilia and found by light microscopy that 2% to 31% of the peripheral blood eosinophils from the patients contained vacuoles, as compared with 5% of normal individuals, and 15% to 28% appeared to have decreased numbers of granules. Similar changes were also seen in patients with transient eosinophilia, and these changes were not observed when the eosinophil counts returned to normal.[19] Electron microscopy showed no granules fused with vacuoles and no vacuoles containing free granules, making the significance of these "holes" uncertain. Weller and Dvorak[20] have postulated that the cytoplasmic vacuolation observed in eosinophils from patients with eosinophilia might be related to increased numbers of lipid bodies because these structures might be solubilized during processing for light microscopy, leaving gaps in the cytoplasm. Only one of our patients had increased numbers of lipid bodies per cell as compared with normal cells, and this patient also had eosinophils with smaller granules than the normodense eosinophils. Our ultrastructural evaluation of hypodense and normodense eosinophils did not demonstrate vacuoles, and it is therefore difficult to interpret such observations in the context of our present findings. However, we can postulate that because the cell size is not significantly different in our HES patients' eosinophils compared with normal eosinophils and because the percentage of cytoplasm occupied by granules is lower in the hypodense group, the "vacuoles" and apparent degranulation observed by light microscopy may represent the increased amount of cytoplasm not occupied by granules due to smaller granules and less total granule content rather than true degranulation. Alternatively, hypodense eosinophils may exhibit morpho-

logic heterogeneity related to the type of underlying disease, duration of disease, or other variables that account for the differences between our observations and those of Spry and Tai.[18,19]

Our data provide a morphological basis for hypodense eosinophils. The findings of consistently smaller individual granules and lower total granule area per cell explain the lower MBP content and point to a critical difference between light and heavy eosinophils from patients with the hypereosinophilic syndrome.

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