

Identification and characterization of monocyte subpopulations from patients with bronchial asthma

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Monocytes are inflammatory cells that accumulate in the airway in asthma. Monocytes constitute a heterogenous cell population in normal subjects. The heterogeneity of monocytes from nine patients with mild asthma and nine normal subjects was studied by means of discontinuous density gradient centrifugation with use of bovine serum albumin. The rate of low-density monocytes recovered from patients with asthma was higher than that obtained from normal subjects. The functional activity of monocyte fractions from asthmatic and control subjects was assessed by using the release of interleukin-1 β (IL-1 β) and the intensity of lysosomal enzymes, such as acid phosphatase and nonspecific esterase. The low-density cells produced less IL-1 β than did cells of higher density in normal subjects. The IL-1 β release was increased in low-density monocytes from subjects with asthma when compared with the same fraction from normal subjects ($p < 0.01$). The low-density monocytes had a higher activity of lysosomal enzymes than did cells of higher density in both asthmatic and normal subjects. Electron microscopic studies showed that low-density monocytes from the subjects with mild asthma appeared to have the morphologic characteristics of activated cells with more vacuoles in this periphery. This study shows that low-density monocytes from subjects with asthma retain the ability to be activated in vivo and in vitro and may orchestrate immune reactions in mild asthma. (J ALLERGY CLIN IMMUNOL 1995;96:230-8.)

Key words: Asthma, blood monocytes, interleukin-1, lysosomal enzyme, electron microscopy

Asthmatic syndrome is characterized by airway inflammation with different cell types, including neutrophils, eosinophils, mast cells, monocytes, and macrophages.^{1,2} Recent research has shown that monocytes and macrophages may play important roles in the pathogenesis of asthma.³⁻⁹ Poston et al.¹⁰ have shown that there are significantly increased numbers of macrophages in biopsy specimens taken from asthmatic airways. Many of these cells have the phenotypic characteristic of blood monocytes, suggesting that there is active recruitment of these cells from the bloodstream into the airways. Monocytes

Abbreviations used

FEV ₁ :	Forced expiratory volume in 1 second
IFN:	Interferon
IL:	Interleukin
SG:	Specific gravity

are precursors of tissue organ macrophages, which are important in inflammatory and immunologic responses.

Monocytes from persons with asthma are activated, and a great number of these cells are sequestered in the lung, where, through the release of cytokines, they participate in the inflammatory process that leads to asthmatic syndrome.^{11,12} Monocytes from persons with chronic stable asthma are more readily stimulated than are monocytes from persons without asthma. Monocytes produce numerous secretory products, such as interleukin (IL)-1. A previous report indicated that monocytes from patients with asthma had

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normal capacity to secrete IL-1 when stimulated with lipopolysaccharide.¹³

Recently, by using albumin gradient or Percoll density gradient centrifugation,^{14,15} some investigators demonstrated morphologic and functional variability within the monocyte subpopulations obtained.¹⁴⁻¹⁶ Monocytes are known to be heterogeneous with regard to morphologic and metabolic, biochemical, and physiologic properties. Monocytes may be sorted on density gradients into subpopulations with different levels of function with respect to the release of IL-1 β or prostaglandins.^{14,16} In addition, alveolar macrophages are a heterogeneous cell population. The cells from persons with asthma have a different distribution of heterogeneous cell populations compared with those from normal persons.¹⁷

These observations led us to hypothesize that a subset of monocyte subpopulations could be activated in the bloodstream in persons with mild asthma as described above for alveolar macrophages and could secrete more IL-1 β . Therefore we examined the density distribution of monocytes, by means of discontinuous bovine serum albumin gradient centrifugation, and determined whether monocyte subpopulations differed in this state of activation, by their ability to secrete IL-1 β after stimulation by lipopolysaccharide or their ability to generate lysosomal enzymes, by means of cytohistochemistry and morphologic examination.

METHODS

Study subjects

As shown in Table I, nine patients (seven men and two women with a mean $\pm$ SD age of 36.0 ± 12.7 years; range, 27 to 66 years) who had clinically stable asthma at the time of the study composed the study group. Asthma was defined as a history of episodic wheezing and reversible airway obstruction characterized by an increase in forced expiratory volume in 1 second (FEV₁) of >15% after inhalation of 40 μ g of procaterol hydrochloride. The severity of asthma was assessed by means of the clinical score of Aas.¹⁸ None of the subjects had smoked within the previous 2 years. Patients were excluded from the study if they had experienced a severe exacerbation of asthma necessitating hospitalization during the month preceding the study. Any drug that may have interfered with the performance of the study was carefully avoided. In particular, subjects were excluded if they had been treated with an oral systemic corticosteroid within 1 month, an inhaled corticosteroid within 7 days, any antihistamine or theophylline within 24 hours, or a β_2 -agonist within 12 hours of testing. The control group was composed of nine normal healthy nonatopic volunteers (seven men and two women with a mean $\pm$ SD age of 35.6 ± 9.9 years; range, 26 to 58

TABLE I. Patient characteristics

Subject No.	Sex	Age (yr)	% Predicted FEV ₁	Aas score	IgE (IU/ml)	Duration of asthma (yr)
1	M	36	93.0	1	889	32
2	M	27	83.2	2	164	25
3	M	25	95.2	1	128	19
4	M	66	75.3	2	644	8
5	M	27	97.3	1	130	21
6	M	31	86.0	1	241	28
7	M	42	95.1	1	186	5
8	F	30	102.7	1	870	2
9	F	40	83.6	1	134	8

years) who did not smoke. No subject had any respiratory tract infection during the month preceeding the study. The study was performed with the informed consent of each subject.

Blood monocyte preparation

Peripheral blood was obtained by means of venipuncture, using heparin (25 U/ml). Mononuclear cells were isolated by the centrifugation of blood samples over Ficoll-Hypaque solutions (Pharmacia Diagnostic, Piscataway, N.J.) at 400 g for 20 minutes at room temperature. The mononuclear cell suspension in Ficoll-Hypaque was washed three times with an equal volume of phosphate-buffered saline solution. Cells were then incubated in tissue culture plates (Falcon 3002, 35 $\times$ 10 mm; Becton Dickinson Labware, Lincoln Park, N.J.) with 5 ml Dulbecco's modified Eagle medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 10% fetal calf serum (Gibco Life Technologies Inc., Grand Island, N.Y.) for 2 hours at 37° C in a humid atmosphere of 95% air and 5% CO₂. The monolayers were then washed three times with warm medium to remove nonadherent cells, such as lymphocytes. Adherent isolated Mo were used either while adherent or after being suspended homogeneously by gentle scraping with a rubber policeman into Dulbecco's modified Eagle medium. They were then characterized with nonspecific esterase staining, and May-Grünwald-Giemsa staining was performed to certify that no other cell was present. The purity exceeded 95%. The viability of the cells, as determined by means of the trypan blue dye exclusion test, was consistently greater than 95%.

Blood monocyte subpopulations preparation by density fractionation

Mo were fractionated on the five-step discontinuous bovine serum albumin (Seikagaku Kogyo Co., Tokyo, Japan) density gradient as described elsewhere.¹⁹ In brief, adherent cells were suspended in 2 ml of 16% bovine serum albumin in Dulbecco's modified Eagle medium, layered over a gradient consisting of 18%, 20%, 22%, and 24% bovine serum albumin, and centrifuged at 900 g for 45 minutes at 4° C. Cells sedimenting

TABLE II. Number of unfractionated Mo and density distribution of Mo

	Number of total monocytes ($\times 10^6/40\text{ml}$ venous blood)	Percentage of fractionated monocytes (%)		
		Low-density monocytes	High-density monocytes	Pellets
Normal subjects	13.2 ± 4.0	6.9 ± 5.7	14.5 ± 6.5	78.6 ± 9.0
Asthmatic subjects	20.2 ± 14.2	$35.3 \pm 24.2^*$	$29.8 \pm 16.6^\dagger$	38.4 ± 27.9

Data represent the mean $\pm$ SD.

* $p < 0.01$ compared with normal subjects.

$^\dagger p < 0.05$ compared with normal subjects.

at the interfaces were divided into the following five fractions: fraction 1, 16%/18% interface; fraction 2, 18%/20% interface; fraction 3, 20%/22% interface; fraction 4, 22%/24%; fraction 5, pellets. The fractions were pooled as follows: low-density monocyte, fraction 1 + 2 + 3 (specific gravity [SG] ≤ 1.062 kg/L); high-density monocyte, fraction 4 ($1.062 < \text{SG} \leq 1.068$ kg/L) and pellets ($1.068 \text{ kg/L} < \text{SG}$). The viability of each fraction exceeded 95% as ascertained by means of trypan blue dye exclusion. The recovery rate varied from 70% to 80%.

Measurement of IL-1 β

Unfractionated and fractionated monocytes ($1.5 \times 10^5/\text{ml}$) were resuspended in Dulbecco's modified Eagle medium, supplemented with 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 10% fetal bovine serum. The cells were stimulated with 10 $\mu\text{g/ml}$ of lipopolysaccharide (*Escherichia coli* 055:B5; Difco Laboratories, Detroit, Mich.). After 24 hours of culturing at 37° C in 5% CO₂, the supernatant and Mo were collected by mechanical agitation and centrifuged to remove the cells. IL-1 β in the supernatant was measured with a commercial ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan). The kit measures specifically native or recombinant human IL-1 β , with no detectable cross-reaction with human IL-1 α , IL-2, IL-3, granulocyte-macrophage colony-stimulating factor or interferon- γ . The minimum detectable concentration was 15.6 pg/ml.

Cytohistochemical assay of lysosomal enzymes

The monocytes were transferred onto poly-D-lysine-coated glass coverslips in a plastic dish and incubated at 37° C for 10 min. They were then fixed with 4% cold paraformaldehyde (pH 7.4) for 5 minutes and maintained in Holt's gum sucrose. They were stained by the naphthol AS-BI phosphate method for acid phosphatase and by the α -naphthyl acetate method for nonspecific esterase.²⁰ Thereafter, they were inspected by means of light microscopy. Mouse peritoneal macrophages were observed simultaneously to determine whether the difference among monocyte subpopulations was statistically significant. Monocytes of these same three subjects were scored visually on a scale of 0 to 3 according to estimates of the number of dying vesicles. Cells were graded for each characteristic by one investigator (T.T.) blinded to the source of monocytes. A score of 0 was given when no

specific characteristic was present, and a score of 3 was given when the estimate of the number present was equal to that of the positive control mouse peritoneal macrophages. A score of 1 or 2 represented gradations between these two extremes. Two hundred cells were counted, and the intensity of staining was determined.

Transmission electron microscopy

Monocyte subpopulations (1 to 2×10^5) were resuspended in 3% glutaraldehyde for a minimum of 1 hour, post-fixed in 1% osmium, dehydrated, and embedded in epoxy resin. By standard methods, thin sections (80 nm) were mounted on uncoated copper grids and stained with uranyl acetate and lead citrate. Thin sections were examined by using a Hitachi HB-600 electron microscope (Hitachi, Tokyo, Japan). A minimum of 70 monocytes from each source and from each of three subjects were examined. Monocyte size was evaluated by measuring the mean diameter of 70 consecutive cells on each section. Morphologic characteristics of monocytes were assessed by means transmission electron microscopy and the number of vacuoles and granules determined.

Statistical analysis

Statistical analysis was performed with the Stat View 4.0 statistics package (Abacus Concepts, Berkeley, Calif.) and a Macintosh Centris 650 computer (Apple Computer, Cupertino, Calif.). All data are expressed as the mean $\pm$ SD. The nonparametric Mann-Whitney U test was used for statistical analysis of the unpaired data obtained regarding the distribution of monocyte subpopulations and the IL-1 secretion study. All paired data were analyzed by means of Wilcoxon's signed-rank test. To analyze the activity of lysosomal enzymes, we used the Spearman test to assess the relationships among the three groups. Variation of the diameter and the number of vacuoles and granules between the three subpopulations was statistically analyzed by using analysis of variance (ANOVA) followed by least significant difference.

RESULTS

Characteristics of the patients

Nine patients had mild asthma (Aas score = 1 to 2). Pulmonary function of the patients, as assessed by means of resting FEV₁, ranged from 75.3% to

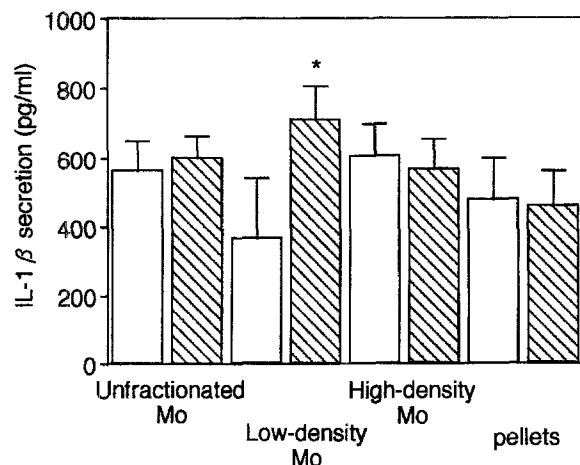


FIG. 1. IL-1 β secretion of supernatants by unfractionated and density-fractionated monocytes of normal subjects (open bars) and patients with asthma (hatched bars). The cells (1.5×10^5 cells/ml) were cultured in the presence of lipopolysaccharide for 24 hours. Cell-free supernatants were then analyzed for immunoreactivity by using the ELISA assay. Mo, Monocytes. (Data represent the mean $\pm$ SD; * $p < 0.01$ compared with control subjects.)

102.7% of predicted values ($90.2\% \pm 8.6\%$ predicted, mean $\pm$ SD) (Table I).

Distribution of monocyte subpopulations

To verify reproducibility, cell separation was repeated in five subjects and similar populations of monocytes were observed (data not shown). There was no significant difference in the total cell count between normal volunteers and patients with asthma (Table II). Patients with asthma had a significantly higher percentage of low-density monocytes compared with normal control subjects ($p < 0.01$) and a correspondingly high percentage of high-density monocytes ($p < 0.05$) (Table II). With regard to the percentage of pellets, there was no difference between normal and asthmatic subjects.

IL-1 β levels in culture medium of monocyte subpopulations

IL-1 β secretion by the unfractionated monocytes and fractionated monocytes after LPS stimulation are summarized in Fig. 1. IL-1 β secretion did not differ between patients' (558.6 ± 91.2 pg/ml, mean $\pm$ SD) and control (601.6 ± 60.2 pg/ml) unfractionated monocytes on stimulation with lipopolysaccharide. When the monocyte subpopulations from normal subjects and patients with asthma were compared, the

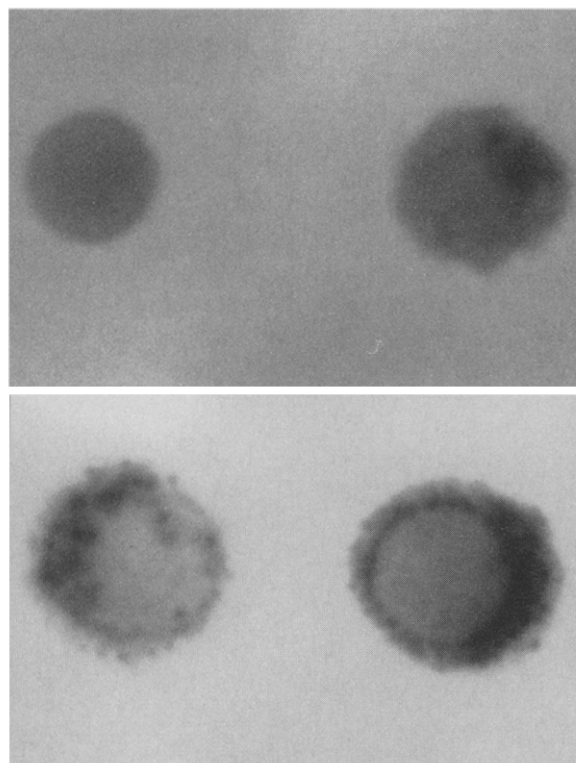


FIG. 2. Cytochemical identification of monocyte subpopulations by the staining of acid phosphatase (top right, score 3; top left, score 1) and nonspecific esterase (bottom right, score 3; bottom left, score 2) (original magnification $\times 200$).

mean IL-1 β concentration in the culture medium of lipopolysaccharide-stimulated low-density monocytes from nine patients with bronchial asthma (708.6 ± 94.1 pg/ml) was significantly higher than that of nine normal subjects (366.4 ± 174.3 pg/ml, $p < 0.01$). No significant difference between normal and asthmatic subjects was found in IL-1 β secretion by high-density monocytes (604.5 ± 92.5 pg/ml and 568.2 ± 85.9 pg/ml, respectively) and pellets (477.0 ± 102.5 pg/ml and 457.2 ± 102.5 pg/ml, respectively).

Activity of lysosomal enzymes

We estimated the intensity of cytochemical staining of lysosomal enzymes, as shown in Fig. 2. When a numerical score was given to cells by a blinded observer, there was correlation between the density fractions and the intensity scores of acid phosphatase in normal subjects ($r_s = -0.70$, $p < 0.020$) and patients with asthma ($r_s = -0.68$, $p < 0.025$) (Fig. 3). Moreover, the intensity score of nonspecific esterase trended to decline with increasing density in normal subjects ($r_s = -0.66$,

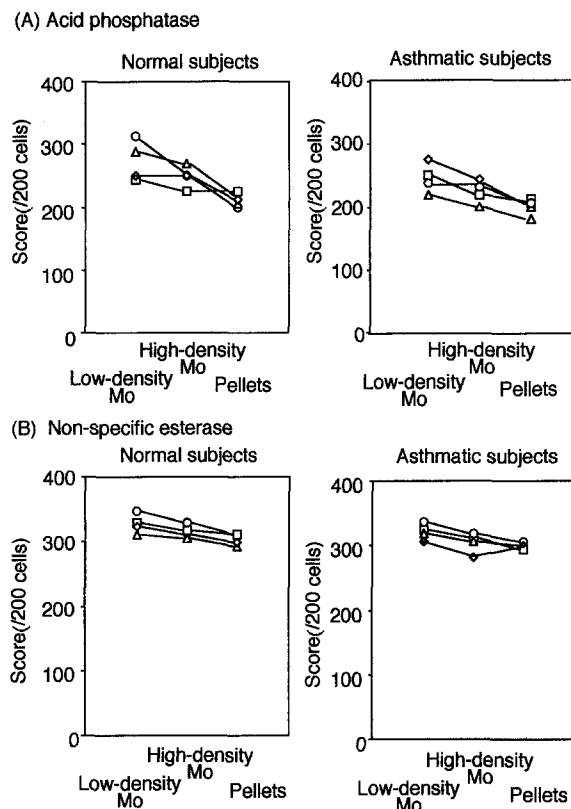


FIG. 3. Heterogeneity of lysosomal enzymes activities of monocyte subpopulations. Intensity of staining was scored on an integer scale of 0 to 3. See Methods for further details. Each symbol represents a different donor. Mo, Monocytes.

$p < 0.029$). The density correlated highly with the intensity score of nonspecific esterase in subjects with asthma ($r_s = -0.66$, $p < 0.029$).

Observation by transmission electron microscopy

The mean size of each monocyte subpopulation was not significantly different between the two groups (Table III). When we estimated the number of granules, the morphologic study showed no difference between asthmatic and normal subjects for each of the three fractions, respectively (Fig. 4). However, the low-density monocytes from subjects with asthma exhibited more numerous vacuoles at the cellular periphery than did other fractions from subjects with asthma ($p < 0.01$, ANOVA analysis) (Table III).

DISCUSSION

Inflammatory cells, such as eosinophils, neutrophils, and alveolar macrophages form a heterogeneous population of cells with respect to a number of parameters, including density. In asthma density fractionation of these cells has demonstrated an

increase in low-density eosinophils,^{22, 23} neutrophils,²⁴ and alveolar macrophages.¹⁷ However, the density distribution of monocytes in subjects with asthma has not been clarified. In this study we demonstrated that patients with mild asthma have higher numbers of low-density monocytes than do normal subjects. Our observations of the density distribution of monocytes from normal subjects are in accord with previous studies,^{14-17, 19} which have demonstrated that most monocyte subpopulations are distributed around pellets ($1.068 \text{ kg/L} < \text{SG}$).

To examine functional heterogeneity of monocytes, researchers have used many different procedures to separate monocytes. Although the use of counterflow centrifugation to identify monocyte subpopulations separated by size has not been concerned with surface receptor activation,²⁵ this procedure was co-eluted with lymphocytes during separation. Elias et al.²⁶ demonstrated that in vitro-cultured monocytes became less dense than those freshly isolated, and it has been suggested that density-defined populations of monocytes may reflect their maturation states. For analyzing the maturation states of monocyte subpopulations in patients with asthma, we undertook the commonly used isolation technique of adherence and separated subsets of monocytes that differ in density. Therefore we investigated whether the low-density monocytes could be stimulated and activated more than monocytes in other fractions with regard to the secretion of IL-1 β and production of lysosomal enzymes.

Activation of monocytes has been observed in the circulation after exercise or allergen provocation.²⁷ Even in the stable stage of the disease monocytes were found to be activated in the bloodstream and to increase the release of oxygen species in patients with asthma.²⁸ Monocytes also became activated or primed, as shown by the enhanced expression of complement receptor and IgG Fc receptors on the cell surface and the increased cytotoxic capacity.²⁷⁻³⁰ Autologous peripheral blood monocytes induced the proliferation of T_{H2}-like lymphocytes released IL-4 and IL-5 selectively.³¹ These activated monocytes are thought to play a role in the pathogenesis of asthma by regulating immunologic responses. Despite the supposition that activated monocytes in patients with asthma possess a higher capacity to secrete IL-1, a previous report indicated that monocytes from patients with asthma have a normal capacity to secrete IL-1 on lipopolysaccharide-stimulation.¹³ Our present findings regarding the secretion of IL-1 β by unfractionated monocytes agree with that report.

It has been shown in animal studies and in normal subjects that low-density monocytes are

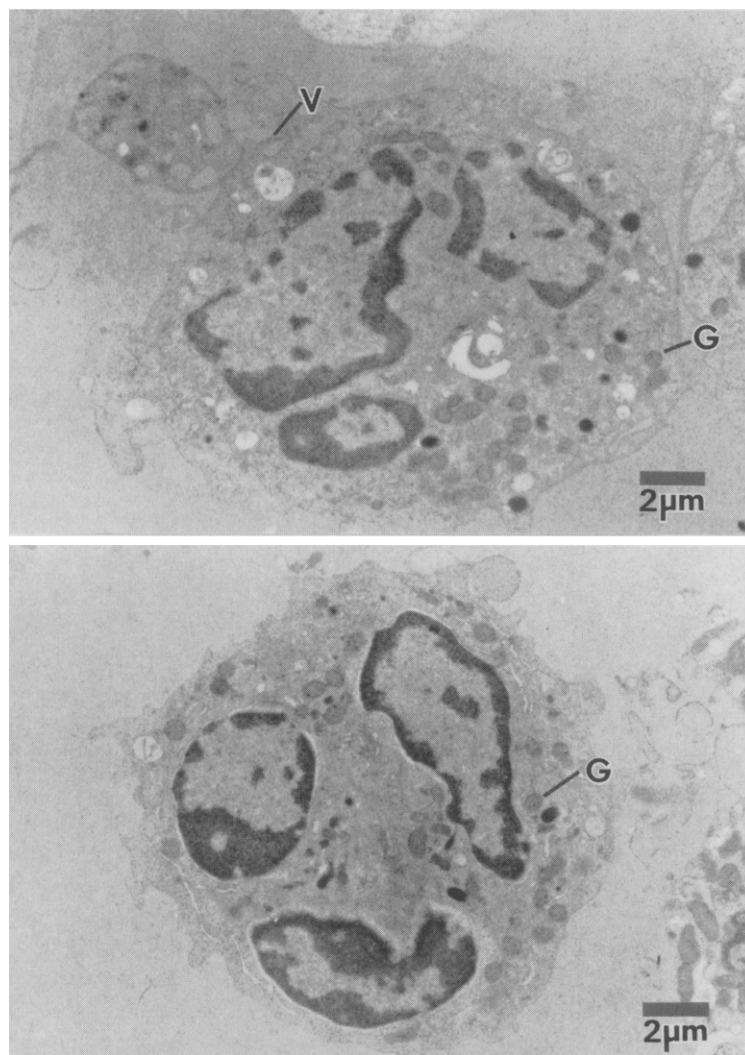


FIG. 4. Electron microscopic study of monocyte subpopulations. *Top*, low-density monocytes from patient with mild asthma; *bottom*, high density monocytes from patient with mild asthma; V, vacuoles; G, granules.

TABLE III. Mean size of cells and assessment of morphologic characteristics

	Low-density monocytes		High-density monocytes		Pellets	
	Normal	Asthmatic	Normal	Asthmatic	Normal	Asthmatic
Diameter (μm)	13.9 ± 1.3	15.0 ± 1.6	13.6 ± 1.3	14.7 ± 1.3	13.5 ± 1.3	14.5 ± 1.3
Vacuoles (numbers/cell)	1.4 ± 1.7	$4.2 \pm 1.2^*$	0.8 ± 1.2	2.6 ± 1.9	0.8 ± 1.2	2.0 ± 1.7
Granules (numbers/cell)	12.3 ± 4.5	16.3 ± 7.0	11.0 ± 3.3	14.6 ± 4.4	11.1 ± 3.3	14.4 ± 5.6

Data represent the mean $\pm$ SD for the number from each subpopulation in a field of 70 monocytes.

* $p < 0.01$ compared with the other density fractions.

less reactive than higher density monocytes with respect to their IL-1 or prostaglandin E_2 release and with respect to their accessory cell function in the immune response.^{26, 32, 33} In agreement with our result, previous reports indicated that in nor-

mal subjects, high-density monocytes release larger amounts of IL-1 β than do low-density monocytes.^{15, 26} We also found that, in subjects with asthma, IL-1 β levels in the culture media of low-density monocytes were significantly higher than

those in the media of low-density monocytes from normal subjects. The secretion of IL-1 β was lowest in high-density monocytes and greatest in low-density monocytes.

On the other hand, our study showed an apparent correlation between density and lysosomal enzymes of monocytes from normal subjects and patients with asthma. The results indicated that monocyte subpopulations differed functionally in their abilities to produce lysosomal enzymes. According to previous observations by Tice et al.³⁴ low-density monocytes produce more acid phosphatase, which is one of the lysosomal enzymes, than do higher density cells. In addition, Picker et al.³⁵ demonstrated an inverse correlation between density fraction and activity of acid phosphatase in normal subjects. These results suggest that low-density monocytes from patients with mild asthma were more sensitive to stimulation than were higher density monocytes.

Our morphologic study demonstrated an absence of any differences in size among the three density fractions. Further, our electron microscopic evaluation of monocytes from stable subjects with asthma showed that most of the low-density monocytes contained significantly more vacuoles, which reflect ruffling and migration, at the peripheral portion of the cells than did the other fractions, whereas they contained a similar number of granules compared with the other fractions. Our data did not demonstrate statistically that there was an inverse relationship between size and density, in spite of admission of their tendency. Cell size of rat alveolar macrophages was found to decrease with increasing cell density.³⁶ The reason that there was no significant relationship between size and density may be that the adherent procedure for 1 hour may make monocytes transform to a spreading shape.

It is only poorly understood why there is a selective increase of low-density monocytes in the bloodstream and why the activities of these cells differ in persons with asthma. These phenomena may be explained by at least two possibilities. First, although the overnight adherence with fetal calf serum is a common procedure, different monocyte subpopulations may be activated respectively after adherence. Adherence is likely to play a pivotal role in the earliest events in monocyte activation. However, expression of mRNA for colony-stimulating factor 1 and IL-1 β after adherence to polystyrene is found to be transitory and restricted to the first few hours after plating.³⁷ Second, recent studies demonstrated that cytokines, such as IL-4 and granulocyte-macrophage colony-stimulating factor,³⁸⁻⁴⁰ made a commitment to differentiation in response to monocytes. IL-4 inhib-

its the ability of human monocytes to produce IL-1.³⁸ Tumor necrosis factor and granulocyte-macrophage colony-stimulating factor can induce differentiation of monocytes to alveolar macrophages and dendritic cells with respect to these morphologic aspects and surface phenotype.^{41, 42} Further, monocyte subpopulations are heterogeneous in response to granulocyte-macrophage colony-stimulating factor, which can stimulate the proliferation and clonal growth of monocytes.³⁹ Therefore in asthma, low-density monocytes, which are a mature stage of cells, may be functionally primed and make an increase in capacity to elaborate IL-1 β by some cytokines.

Monocytes, whose numbers were also found to be increased in the airway mucosa, are known to be capable of migrating from the blood to the lung, where they mature and acquire more stimulatory activity. Monocytes are also present in the lung and, indeed, most alveolar macrophages and dendritic cells originate from monocytes.^{43, 44} However, only a small fraction replicate *in loco*.⁴⁵ Bellini et al.³¹ described an increased number of dendritic cells in bronchial biopsy specimens from persons with asthma and an enhanced antigen-presenting capability of dendritic cells.³¹ Asthmatic bronchi are infiltrated with inflammatory cells with a monocytic phenotype, suggesting recruitment of monocytes to the lung in asthma.¹⁰ Our observation supports that the hypothesis that low-density monocytes, which are at maturation stage in the bloodstream, migrate into the airways. However, further experimentation will be required to definitively prove this hypothesis.

Elevations in IL-1 β have been found in bronchoalveolar lavage fluid removed from patients with asthma.⁴⁶ There are many mechanisms by which IL-1 β may induce or amplify inflammatory reactions in the airways. IL-1 β may act directly, by promoting the expression of adhesion molecules on endothelial cells,⁴⁷ thereby affecting leukocyte migration. Consequently, IL-1 may have a role in the pathogenesis of asthma by causing the accumulation of leukocytes into the airway mucosa. In a guinea pig model of pulmonary anaphylaxis, IL-1ra, a specific IL-1 receptor antagonist, inhibits antigen-induced airway hyperactivity to substance P and inflammatory cell influx in the sensitized guinea pig, which suggests that this cytokine may have a significant role in the pathogenesis of pulmonary anaphylaxis.⁴⁸ If low-density monocytes migrated into the airways in persons with mild asthma, they elaborate most IL-1 and have the potential to contribute to the regulation of airway inflammation in asthma.

In conclusion, patients with mild asthma have a

different distribution of monocytes compared with normal subjects. Low-density monocytes are activated in the bloodstream of normal subjects and persons with asthma secrete more IL-1 β and possess more vacuoles. Therefore our data suggest that the subset of monocyte subpopulations may orchestrate inflammatory responses and control the immunologically mediated processes involved in the early recruitment of these cells into the lung.

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