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Blood monocytes of untreated asthmatics exhibit some features of tissue macrophages

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SUMMARY

Airway macrophages are activated in asthmatic subjects. Peripheral blood monocytes from these subjects present some functional features of activation, but their membrane markers are not known. Recently a new subtype of blood monocytes, $CD14^+/CD16^+$, has been identified which possesses the characteristics of tissue macrophages. A study was carried out on nine normal subjects and 11 untreated asthmatics having variable severities of the disease to examine the phenotypic characteristics of monocytes. CD14, CD16, HLA-DR, CD11a, CD11b, CD44 and CD54 were studied using double fluorescence flow cytometry since these antigens have been defined in the $CD14^+/CD16^+$ monocytes. The functional activation of monocytes was examined using the release of superoxide anion. The co-expression of CD14 and CD16 by monocytes in terms of percentage and mean fluorescence intensity was significantly higher in asthmatics ($P < 0.002$ and $P < 0.0001$, respectively, Mann–Whitney *U*-test). There was no difference for the other membrane markers between asthmatics and normal subjects. Superoxide anion release was significantly increased in asthmatic subjects ($P < 0.01$). This study shows that most blood monocytes of asthmatics are $CD14^+/CD16^+$ and are likely to present features of tissue macrophages.

Keywords monocyte CD14 CD16 asthma flow cytometry

INTRODUCTION

Airway macrophages are among the cells involved in the chronic inflammatory processes of bronchial asthma. In bronchial biopsies of asthmatic patients, the number of macrophages is increased in comparison with control subjects, and these cells bear both HLA-DR and blood monocyte markers [1,2]. In the airways, macrophages proliferate to a similar extent in both asthmatic and normal subjects [3], suggesting that the greater number of cells of the monocytic lineage found in biopsies of asthmatic patients is due to an increased recruitment from blood monocytes. Alveolar macrophages (AM) from asthmatic subjects differ functionally and metabolically from those obtained from normal subjects. The expression of intercellular adhesion molecule-1 (ICAM-1) (CD54) and LFA-1 (CD11a) is increased on AM [4] of asthmatic patients compared with those from normal subjects, and these cells release larger amounts of cytokines and eicosanoids [5,6]. Moreover, peripheral blood monocytes from asthmatic patients appear to be activated, since they release increased levels of superoxide anion [7].

While it is known that there is great heterogeneity in the function and phenotype of tissue macrophages there is little information regarding the heterogeneity of monocytes. Monocyte phenotype varies according to the state of maturation of the cells [8,9]. The expression of the CD14 antigen increases with maturation, is strongly expressed on monocytes and is reduced in AM [10]. There is little expression of the CD16 antigen on peripheral blood monocytes, but the novel subset of $CD14^+/CD16^+$ monocytes recently described exhibits some features of tissue macrophages, such as increased expression of HLA-DR and low levels of CD11b [11].

A study was carried out involving nine healthy individuals and 11 asthmatic patients not treated with inhaled steroids and with variable severity of the disease, to assess whether there was activation of blood monocytes and if these cells exhibited features of tissue macrophages. The functional activation of monocytes was assessed by measuring superoxide anion release. The phenotypic characteristics of monocytes were studied using flow cytometry, and the expression of CD14,

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Table 1. Results of flow cytometry analysis

		Healthy subjects	Asthmatics	*P*
CD14	%	29·5 (20–34)	29·5 (16–53)	
	MFI	25 (20–34)	33 (26–41)	< 0·003
$CD14^+HLA\text{-}DR^+$	%	81 (31–96)	92 (75–98)	
	MFI	25 (12–54)	43·5 (20–85)	< 0·04
$CD14^+CD16^+$	%	45 (29–73)	96 (50–99)	< 0·002
	MFI	10 (9–15)	37 (16–66)	< 0·0001
$CD14^+CD11a^+$	%	90 (67–100)	97 (90–100)	
	MFI	6 (5–11)	8 (6–10)	
$CD14^+CD11b^+$	%	100	100	
	MFI	34 (23–48)	45 (34–67)	
$CD14^+CD54^+$	%	100	100	
	MFI	4 (2–8)	5·5 (3–31)	
$CD14^+CD44^+$	%	92·5 (82–100)	97 (64–100)	
	MFI	5 (3–12)	7 (5–12)	

Results are expressed in medians and range. Statistical analysis by Mann–Whitney *U*-test. Using Bonferroni's correction only values with $P < 0{\cdot}008$ are considered significant.

MFI, Mean fluorescence intensity.

CD16, HLA-DR, CD11a, CD11b, CD44 and CD54 were examined.

PATIENTS AND METHODS

Subjects

Eleven asthmatic subjects (age 22–69 years, median 43·0 years) were enrolled in the study. Asthma was defined according to the criteria of the American Thoracic Society [12]. All had a reversible airways obstruction assessed by an increase of 12% of FEV_1 and an absolute value of 200 ml after inhalation of 200 μg salbutamol [13]. The severity of asthma was quoted according to the score of Aas [14], which was previously found to be correlated with the severity of asthma [15]. The severity of asthma ranged from mild to severe (forced expiratory volume in 1 s (FEV_1) FEV_1 40–100%, median 84·0% of predicted). Nine normal volunteers (age 23–63 years, median 34·5 years) were enrolled in the study. None of them had any previous history of lung or allergic disease, and none of the subjects tested was a smoker. No subject had received any drug which could alter test results [15]. In particular, they had not taken any systemic corticosteroids during the past 2 months and inhaled corticosteroids the previous month. Atopy was studied as previously described in detail [15[.

Separation and purification of mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation from 15 ml of heparinized blood. The blood was diluted in RPMI (1:3) and layered onto Ficoll–Hypaque (Kabi-Pharmacia Diagnostics, Uppsala, Sweden) [16]. The cells which formed a band at the interface were aspirated, washed three times in PBS, and used for flow cytometry analysis or superoxide anion release.

Immunofluorescence staining and flow cytometry of monocytes

The expression of the markers was determined by a double staining procedure. Antibodies were used at a concentration of 1:20, and staining was performed over a period of 10 min. For determination of CD11b or HLA-DR markers, we used an FITC-conjugated anti-CD14 MoAb (Becton Dickinson, Sydney, Australia) and a second MoAb conjugated to PE and reacting against CD11b or HLA-DR (PE-conjugate; Becton Dickinson). For determination of CD16 expression, the cells were first stained with an anti-CD16 MoAb (CLB, Amsterdam, The Netherlands) followed with a PE-conjugated rat anti-mouse κ chain antibody (Becton Dickinson) and then with the FITC-conjugated anti-CD14 MoAb. For the determination of CD11a and CD44 antigens, we used a PE-conjugated anti-CD14 MoAb (Serotec, Oxford, UK) and FITC-conjugated anti-CD11a and CD44 MoAb (Becton Dickinson). Monocytes were selected by side scatter and expression of CD14. Only $CD14^+$ cells were analysed for the expression of other markers. Flow cytometry was performed with the 488-nm line of an argon ion laser EPICS Profile (Coulter Electronics, Miami, FL). A fluorescence histogram of at least 5000 counts was collected from each sample. The mean fluorescence intensity (MFI) was measured on a logarithmic scale. The isotype control antibodies mouse IgG1–FITC and mouse IgG1–PE (Becton Dickinson) were used as non-specific staining. When the test and control histograms were discrete, a marker was set on the control histogram such that 1% of the cells were to the right of this channel marker.

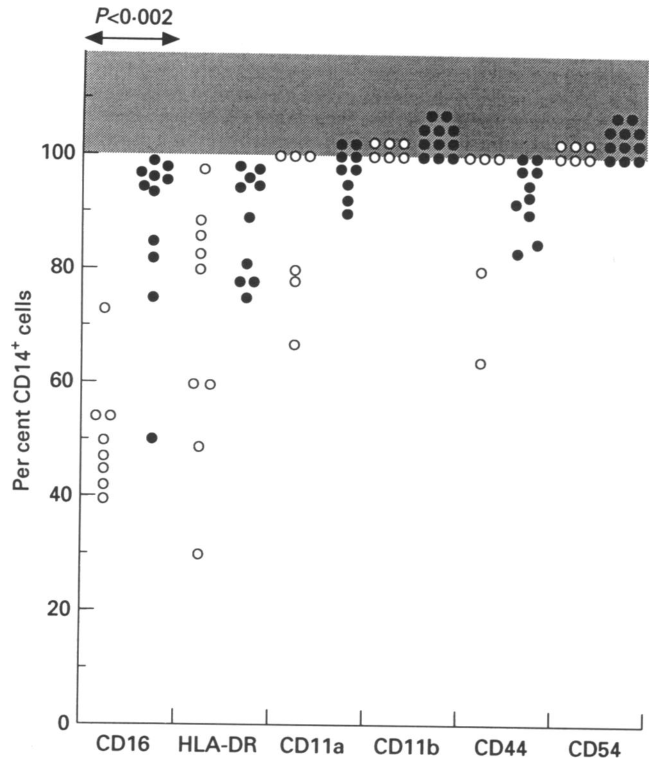

Fig. 1. Percentage of $CD14^+$ cells expressing membrane markers in asthmatic (●) and normal subjects (○). Statistical analysis by Mann–Whitney *U*-test.

Superoxide anion release by monocytes

Cells recovered from the interface of the Ficoll gradient were allowed to adhere on 96-well plates at a concentration of 150 000 cells/well for 60 min at 37°C in a 5% CO_2 atmosphere

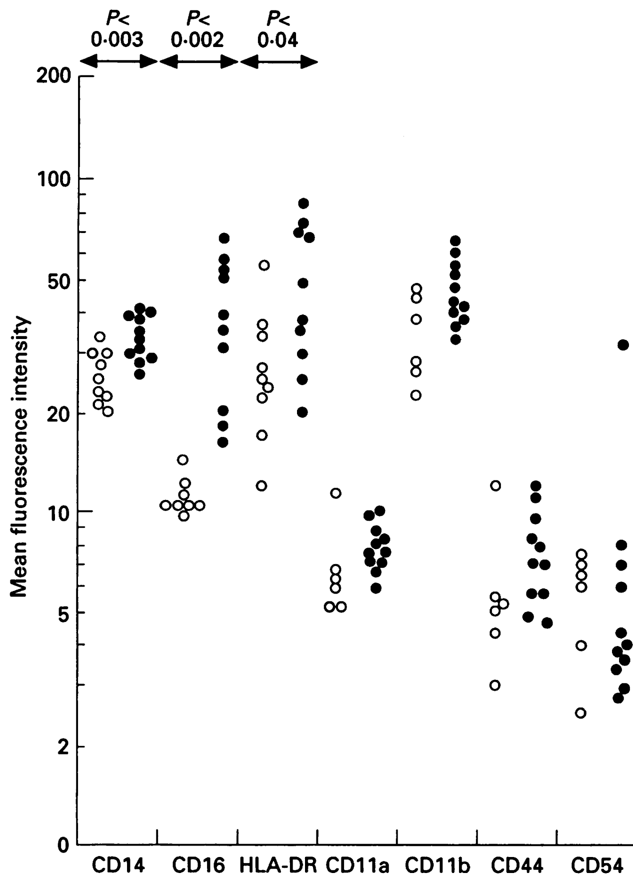

Fig. 2. Expression of membrane markers on peripheral blood monocytes of asthmatic (●) and normal subjects (○). Statistical analysis by Mann–Whitney *U*-test.

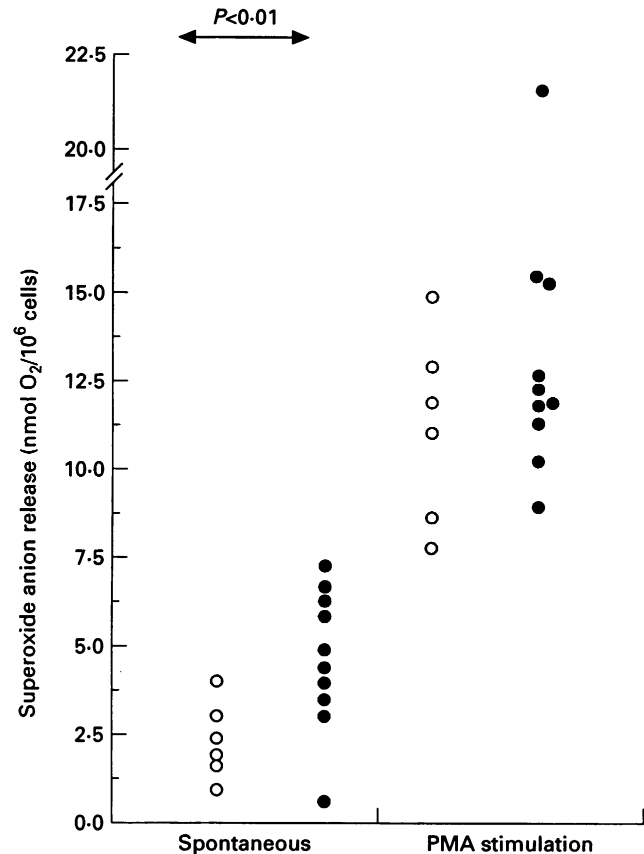

Fig. 3. Release of superoxide anion by peripheral blood monocytes of asthmatic (●) and normal subjects (○). Statistical analysis by Mann–Whitney *U*-test. PMA, Phorbol myristate acetate.

in order to remove the remaining granulocytes. Although it is not possible to enumerate neutrophils in the 96-well plate, we attempted to determine whether some neutrophils may have adhered to plastic by enumerating them before and after adherence. In gradients in which neutrophils were over 5% before adherence and when the difference between both enumerations was over 20%, the sample was not taken into consideration. Superoxide anion release was studied in the absence and presence of phorbol myristate acetate (PMA; 20 ng/ml) using the reduction of ferri-cytochrome C (Sigma, St Louis, MO) as an index of superoxide anion release as described by Pick & Mizel [17]. The results are expressed in nmol of superoxide anion release by 10^6 cells.

Statistical analysis

The Mann–Whitney correlation with Bonferroni's correction was used. Results are expressed as medians and range.

RESULTS

Flow cytometry of monocytes

Results of flow cytometry are shown in Table 1 and Figs 1 and 2. The percentage of $CD14^+/CD16^+$ was significantly greater in asthmatics ($P < 0{\cdot}002$, Mann–Whitney *U*-test) than in normal subjects. The percentage of $CD14^+HLA\text{-}DR^+$ cells was slightly greater in the asthmatic group, though there was no difference between the groups for the other four markers. The MFI was significantly greater in asthmatic subjects for $CD14^+$, $CD14^+CD16^+$ and $CD14^+HLA\text{-}DR^+$ ($P < 0{\cdot}003$, $P < 0{\cdot}0001$ and $P < 0{\cdot}04$, respectively). However, using Bonferroni's correction the MFI difference between asthmatic and control subjects for $CD14^+HLA\text{-}DR^+$ was no longer significant. There was no difference for the other membrane markers. There was no correlation between the severity of the disease and the expression of phenotypic markers. There was no difference between asthmatics who had a history of allergy and those who were not atopic.

Superoxide anion release by monocytes

Superoxide anion release is shown in Fig. 3. The spontaneous release was significantly increased in asthmatic patients ($P < 0{\cdot}01$) in comparison with normal subjects, but PMA-induced release was similar in both groups.

DISCUSSION

The results of this study show that there is an increase in the $CD14^+/CD16^+$ subset of monocytes which exhibit features of tissue macrophages in asthmatic subjects, and that these cells are functionally activated since there is an increase in spontaneous superoxide anion release and in the expression of HLA-DR.

We studied the phenotypic and functional characteristics of blood monocytes from asthmatics since it has been suggested that there is an increased recruitment of monocytes into the airways of asthmatic patients, albeit indirectly [2,3], and these cells have been shown to be functionally activated [7]. The membrane markers were selected since: (i) they represent characteristic antigens of monocytes (CD14) [18] or macrophages ($CD14^+/CD16^+$) [11]; (ii) their expression is increased during the inflammatory process on cells of the monocytic lineage of asthmatics (CD11a, CD44 and CD54) [2,4]; (iii) they are involved in the phenotypic characterization of the novel $CD14^+/CD16^+$ subset of monocytes (CD11b, HLA-DR) [11]; (iv) they are increased in activated AM in pulmonary disorders (HLA-DR) [19]. Flow cytometry was used to assess the expression of surface markers, since we felt that this method had several advantages over the alkaline-phosphatase anti-alkaline-phosphatase (APAAP) technique. First, very few cells are required for flow cytometry. Second, a double staining procedure can be used, allowing one to compare cells expressing two different markers in the same sample. Third, this method is quantitative in that the intensity of fluorescence is indicative of the amount of each marker expressed. Fourth, flow cytometry is more sensitive than APAAP [20]. The percentage of $CD14^+$ cells in each preparation shows that isolation techniques were similar and that comparisons can be made.

The $CD14^+CD16^+$ monocytes of asthmatics observed in this study are not completely similar to those reported by Ziegler-Heitbrock *et al.* [11]. In particular, we observed that the expression of CD11b and CD54 was low and that of HLA-DR relatively high. However, the MFI of CD14 cells was found to be greater in these cells despite the fact that in AM CD14 is decreased. This would suggest that the subset of blood monocytes described in this study express some, but not all features of AM. The elevation of CD14/CD16 monocytes in asthmatic patients probably represents a transitional maturation stage rather than a functionally specialized subtype. None of the asthmatic subjects included in the study had received inhaled corticosteroids recently, and the severity of their disease ranged from mild to severe, but there was no correlation between severity of disease and expression.

The release of superoxide anions was measured by the reduction of ferricytochrome C [17], since using this technique we have previously demonstrated a difference between the levels of superoxide anions released from monocytes obtained from asthmatic and normal subjects (unpublished data). This technique does not take into account peroxidase activities of the cells that may be modulated by their maturation [21]. In addition, this method is simple, reproducible, and requires relatively few cells. However, we carefully avoided the presence of neutrophils that can release large amounts of superoxide anions [22]. Since AM release greater amounts of superoxide anion than monocytes [23], there exists a maturation of monocytes in asthma as these cells released increased amounts of superoxide anions by comparison with those of normal subjects. This result is in agreement with the increase in the $CD14^+/CD16^+$ phenotype of monocytes in asthma. We did not measure other markers such as leukotrienes and peroxidase, since the number of cells recovered was insufficient for the measurement of a range of mediators.

The reasons for the maturation of monocytes in asthma are unclear, but may be related to chemotactic factors present in the peripheral blood of asthmatic patients and demonstrated for eosinophils and neutrophils [2,4]. Alternatively, cytokines present in the serum of asthmatics may have increased the oxidative burst of the cells [25–27]. The maturation of monocytes in asthma may be of importance for their increased recruitment into the airways [28,29]. We did not study all adhesion molecules involved in the rolling and adhesion of cells to the endothelium, since it is likely that there is an increased expression of molecules on the surface of the cells and an increased affinity for their ligands [29].

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