THE goal of our study was to assess the chemotactic activity for eosinophils (ECA) and neutrophils (NCA) and histamine releasing activity (HRA) in crude supernatants of mononuclear cells in monosensitized atopic asthmatics and healthy controls. Chemotactic activity for ECA and neutrophils was measured in supernatants of cultured mononuclear cells with modified Boyden's chamber and HRA was assessed on healthy donor basophils. With respect to ECA generation two distinct subgroups of subjects were distinguished: releasers [ECA (+)] and non-releasers [ECA (–)]. In atopic and non-atopic ECA (+) the mean ECA index was 3.78 ± 0.49 and 2.47 ± 0.27 respectively (*P* > 0.05). Supernatants from the remaining subjects (seven of 22 atopic and five of 11 non-atopic) did not express ECA, but revealed significant inhibitory activity for chemokinesis of eosinophils (mean chemotactic index 0.25 ± 0.16 and 0.48 ± 0.22 for atopic and non-atopic non-releasers respectively). Stimulation with antigen of MNC from atopic and with PHA from non-atopic ECA (–) restored cells ability to release ECA. Sephadex gel chromatography revealed that supernatants of MNC contained chemotactic and chemokinesis inhibitory activity in different fractions. The spontaneous productions of NCA and HRA by mononuclear cells was similar in ECA releasers and non-releasers, although the HRA was higher following stimulation with PHA in the non-atopic ECA (+) subgroup. Our study demonstrated, for the first time, that MNC are capable of generating not only chemotactic activity but also chemokinesis inhibitory activity for eosinophils.

Key words: Allergic inflammation, Eosinophil chemotactic activity, Eosinophil chemokinesis inhibition, Neutrophil chemotactic activity, Histamine releasing activity

The release of eosinophil chemotactic activity and eosinophil chemokinesis inhibitory activity by mononuclear cells from atopic asthmatic and non-atopic subjects

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Introduction

Bronchial asthma is a chronic inflammatory process, characterized by the influx and accumulation of eosinophils, neutrophils, lymphocytes and monocytes/macrophages in the airway mucosa.[1] An increased number of eosinophils was observed in bronchial mucosa and in bronchio-alveolar lavage (BAL) in patients with seasonal and perennial asthma.[2] The eosinophilic influx after allergen provocation is accompanied by the increase of eosinophils chemotactic activity (ECA) in BAL.[3,4] Similarly natural exposure to allergen induces the increase of the ECA in bronchoalveolar lavage and in serum.[3] Our earlier studies also demonstrated the presence of ECA in the serum of patients with atopic asthma as well as chemotactic activity for neutrophils (NCA) in nasal lavage in patients suffering from pollinosis.[5,6] These data suggest that mechanisms initiated as a response to allergen leading to release of chemotactic molecules, which are responsible for the selective influx of eosinophils to the lungs.

Mononuclear cells play an important role in the development of allergic inflammation by the generating of cytokines with proinflammatory properties, thus participating actively in the generation of the effector phase of allergic inflammation.[7–12] Several cytokines, e.g. RANTES or MCP, have eosinophil chemotactic activity and additionally may cause basophil degranulation and release histamine.[13–15] Other mononuclear cell-derived cytokines (e.g. IL–8 and MCP–1) have NCA and HRA.[16–18] Acting in concert with other chemotactic agents originating from mastocytes and basophils (e.g. PAF, LTB4) these cytokines induce the influx of cells into the site of the allergic reaction. Most studies have focused on the measurement of the release of clearly defined cytokines by MNC and on the effect of these cytokines in isolated biological systems.[19–21] However, activated inflammatory cells release, at the same time or

sequentially, several mediators, and their biological effect is the result of complex and not completely understood interactions. Thus, measurement of biological activity of 'crude' supernatants of antigen-stimulated cells may provide observations more relevant to *in vivo* situations, than measurement of a single mediator or a cytokine.

The aim of our study was to investigate the ability of MNC to spontaneous and antigen-induced generation of chemotactic activity for eosinophils and neutrophils as well as HRA in a group of asthmatic patients, allergic to the house dust mite antigen Der p I. The results obtained in the asthmatic patients were compared with the control group of healthy individuals.

Materials and Methods

Patients

The study included 22 patients (mean age 35 ± 7.19 years; 12 female and 10 men) with perennial atopic bronchial asthma and clinically dominating allergy to house dust mites. All patients had positive skin prick tests with Der p I allergen. No patient was on inhaled oral corticosteroids for at least 2 weeks before the study. Methyloxantins or short-acting antihistamines were stopped at least 72 h before obtaining their blood samples. A control, non-atopic group comprised 11 healthy subjects without any complaints concerning the respiratory tract and with negative skin prick tests to a battery of inhalant allergens.

MNC isolation and culture

Mononuclear cells were isolated with Boyüm's method.[22] In brief, 20 ml of heparinized venous blood was mixed with PBS in 1:3 proportion, then carefully stratified on 'Lymphoprep' gradient (Pharmacia) and centrifuged at $400 \times g$ for 20 min. The ring, which formed on the borderline of the phases, was carefully collected and rinsed in phosphate buffer pH 7.4 without Ca^{++} and Mg^{++} as well as 0.036% glucose so that the number of cells equalled 2×10^6 ml. Next, the cells were cultured according to Theuson's method.[23] Culture medium consisting of PBS with Ca^{++} and Mg^{++} or 0.036% glucose and 0.1% bovine albumin was used. Der p I allergen in the concentration 10^{-2} µg/ml (in the asthmatic subjects) or PHA mitogen in concentration 25 µg/ml (in healthy subjects) were added to cell suspension and the samples were preincubated for 4 h in the incubator with 5% CO_2 and 95% humidity at 37°C. Then the cells were centrifuged for 20 min $400 \times g$, washed twice in PBS without Ca^{++} and Mg^{++}, suspended in the primary medium volume and cultured for 24 h in the same conditions. Cells cultured in the medium alone (spontaneous cultures) served as a control. Then the

samples were centrifuged for 20 min at $400 \times g$, supernatants dialysed in PBS for 24 h and frozen until ECA, NCA and HRA activities were measured.

Measurement of ECA and NCA

Granulocytes from a healthy donor, previously tested for cells chemotactic activity, were isolated using Boyüm's method.[22] Chemotaxis was measured by applying modified Boyden's method using a 48-well chamber (Neuroprobe; Bethesda, MD, USA) and Millipore filters PVP-free with pore diameter 5 µm and 10 µm thick.[24,25] To the specified wells at the bottom of the chamber 30 µl of the studied supernatants were added in triplicate, then the filter was applied and the whole chamber assembled. To the wells of the upper part of the chamber 25 µl of the previously isolated granulocytes suspended in culture medium 2×10^6 ml were added. The chamber was incubated for 90 min in the incubator with 5% of CO_2 and 95% humidity at 37°C. Subsequently the chamber was disassembled, the filter preserved and stained with Wright–Giemsa and Chromotrope 2R. The filters were assessed in light microscope at 500× magnification by an investigator who was unaware of the design of the experiment. Chemotactic activities in the studied supernatants were estimated by counting granulocytes (eosinophils or neutrophils) in five standardized viewing areas. Chemotaxis was expressed as chemotactic index CI = B/A; B being the number of cells migrating to the supernatant, and A the number of cells migrating to the control medium.

Measurement of HRA

To evaluate the supernatants derived HRA the method of Siriganian was used.[26] Basophils from the healthy donor were isolated as previously described[13] and to 100 µl of the cell suspension (2×10^6 cells/ml), 400 µl of the examined supernatant was added. The samples were incubated at 37°C for 30 min while carefully stirred, and then cells were cooled rapidly to 4°C and centrifuged for 8 min (1500/min, temperature 4°C). The supernatants (containing released histamine) and the sediments were secured by freezing. Histamine was assessed with the spectrofluorometric method.[26] The read-outs (in relation to the standard curve for histamine) were carried out on an LS 50 spectrofluorometer (Perkin-Elmer). HRA was expressed as the percentage of the released from basophils histamine according to the following formula:

$$\%HRA = \frac{\text{Histamine in the basophils supernatant} \times 100}{\text{Histamine in the basophils supernatant} + \text{histamine in the sediment}}$$

Partial purification of the chemotactic activity by gel column chromatography

The approximate molecular weight of the chemotactic activity was determined. Supernatants obtained from MNC (from three asthmatic and three healthy subjects) were stratified on a column containing Sephadex gel G–75 (by Pharmacia) at flow rate of 12 ml/h. The mononuclear cell culture supernatant fluid was eluted with PBS. Two-ml fractions were collected and analysed in duplicate for ECA, NCA or HRA. A kit (Sigma) for molecular weight as standard for column chromatography was used. Protein concentration in each fraction was measured at absorbency 280 nm.

Statistical analysis

The statistical analysis was performed with Student's *d*-test and *t*-test, preceded by evaluation of normality with *F*-test. In all cases, a *P* value less than 0.05 was considered significant.

Results

ECA release by mononuclear cells from atopic patients

Significant ECA was measured in the supernatants of mononuclear cells isolated from 14 out of 22 asthmatic patients and the average chemotactic index for this subgroup [ECA (+)] was 3.78 ± 0.49. Supernatants obtained from the remaining eight patients not only did not express chemotactic activity, but clearly inhibited the migration (chemokinesis) of eosinophils. The average value of the chemotactic index in this group was 0.25 ± 0.16. This subgroup was denoted as non-releasing ECA [ECA (−)].

Stimulation of the mononuclear cells from atopic asthmatic patients with Der p I antigen did not affect the mean ECA for the whole group (*n* = 22; before stimulation the mean chemotactic index was 2.5 ± 0.48; after stimulation 2.45 ± 0.69; *P* > 0.05). However in the ECA (+) subgroup, antigen stimulation considerably decreased the activity in 10 of 11 patients and the mean ECA index was 1.98 ± 0.49 (*P* < 0.05). In contrast, in the ECA (−) subgroup, after stimulation with antigen, the increase in eosinophil chemotactic activity was revealed in seven out of eight examined patients (the mean value of chemotactic index increased to 3.73 ± 1.73; *P* < 0.05) (Fig. 1).

ECA release in healthy individuals

The mean ECA in healthy individuals was similar to mean ECA in atopic patients (*P* > 0.05). In the group of healthy individuals significant ECA was present in six out of 11 examined supernatants [ECA (+)] and the remaining supernatants [ECA (−)], had suppressive effect on chemokinesis of eosinophils. The mean index in the ECA (+) and ECA (−) subgroups were 2.44 ± 0.27 and 0.48 ± 0.22 respectively (Fig. 2). Stimulation of MNC with Der p I antigen did not affect ECA generation in control patients (data not shown).
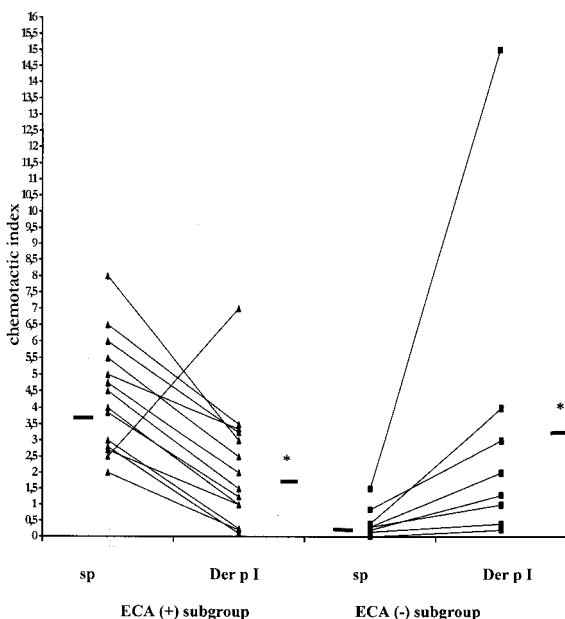

FIG. 1. ECA generation by mononuclear cells (MNC) from atopic asthmatic patients. *P<0.05 compared with spontaneous release. Sp.–spontaneous release; Der p I–stimulated release with Der p I. Mean value.
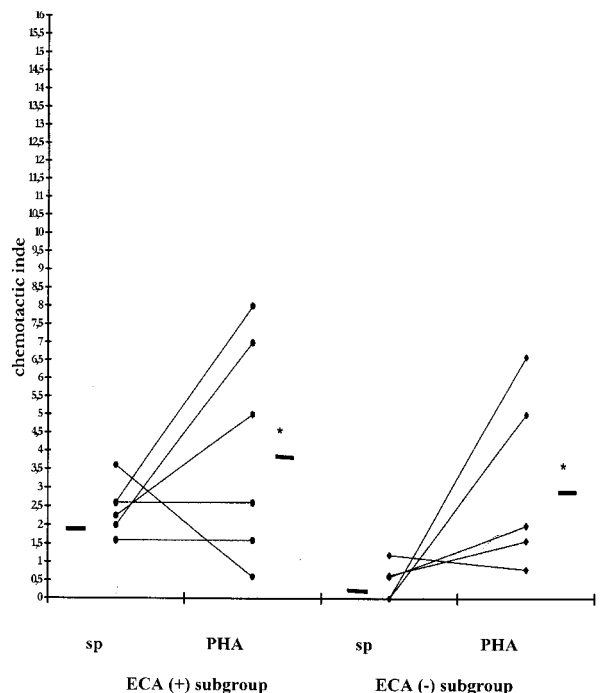

FIG. 2. ECA generation by mononuclear cells (MNC) from healthy non-atopic subjects. *P<0.05 compared with spontaneous release. Sp.–spontaneous release; PHA–stimulation release. Mean value.

Table 1. Generation of NCA and HRA by mononuclear cells from control non-atopic subjects and from asthmatic patients sensitive to Der p I antigen; *$P < 0.05$ compared with non-stimulated cells; ‡$P < 0.05$ compared with ECA (+) subgroup

NCA index	Control non-atopic subjects		Asthmatic patients	
	ECA (+) *n*=6	ECA (−) *n*=5	ECA (+) *n*=14	ECA (−) *n*=8
Spontaneous	1.85 ± 0.14	1.41 ± 0.26	2.48 ± 0.27	2.11 ± 0.22
Stimulated[a]	1.50 ± 0.08	1.40 ± 0.21	2.27 ± 0.20	2.15 ± 0.13
HRA %				
Spontaneous	20.80 ± 1.76	21.99 ± 2.98	30.91 ± 1.84	27.09 ± 3.54
Stimulated[a]	17.75 ± 1.63	31.76 ± 1.65*‡	41.77 ± 1.50*	36.80 ± 1.46

[a] The cells were stimulated with PHA or Der p I antigen in non-atopic and atopic subjects respectively.

After stimulation with phytohaemagglutinin (PHA) the mean value of ECA index increased to 4.13 ± 1.22 for the ECA (+) subgroup and 3.19 ± 1.1 for the ECA (−) subgroup ($P < 0.05$) (Fig. 2).

NCA and HRA release from MNC atopic patients

Mononuclear cells supernatants from all atopic patients contained significant neutrophil chemotactic activity (NCA) but there were no significant differences between the values of chemotaxis index in the ECA (+) or ECA (−) subgroups (mean 2.48 ± 0.27; 2.11 ± 0.22 for both groups respectively) (Table 1). Stimulation of MNC with Der p I antigen did not affect neutrophil chemotactic activity neither in the ECA (+) nor ECA (−) groups.

A significant HRA was found in MNC supernatant of 22 asthmatic patients (mean 29.33% ± 1.80) with no difference between ECA (+) or ECA (−) patients ($P > 0.05$). There was an increase in the percentage of histamine release from basophils in response to supernatants obtained from antigen stimulated cells comparing to spontaneous culture supernatants, however only the increase in the ECA (+) group was significant ($P < 0.05$) (Table 1).

NCA and HRA in the MNC supernatants of healthy individuals

Mononuclear cells from the healthy subjects generated neutrophil chemotactic activity (NCA) and the mean chemotaxis index was similar to atopic patients ($P > 0.05$). There was no difference in NCA between
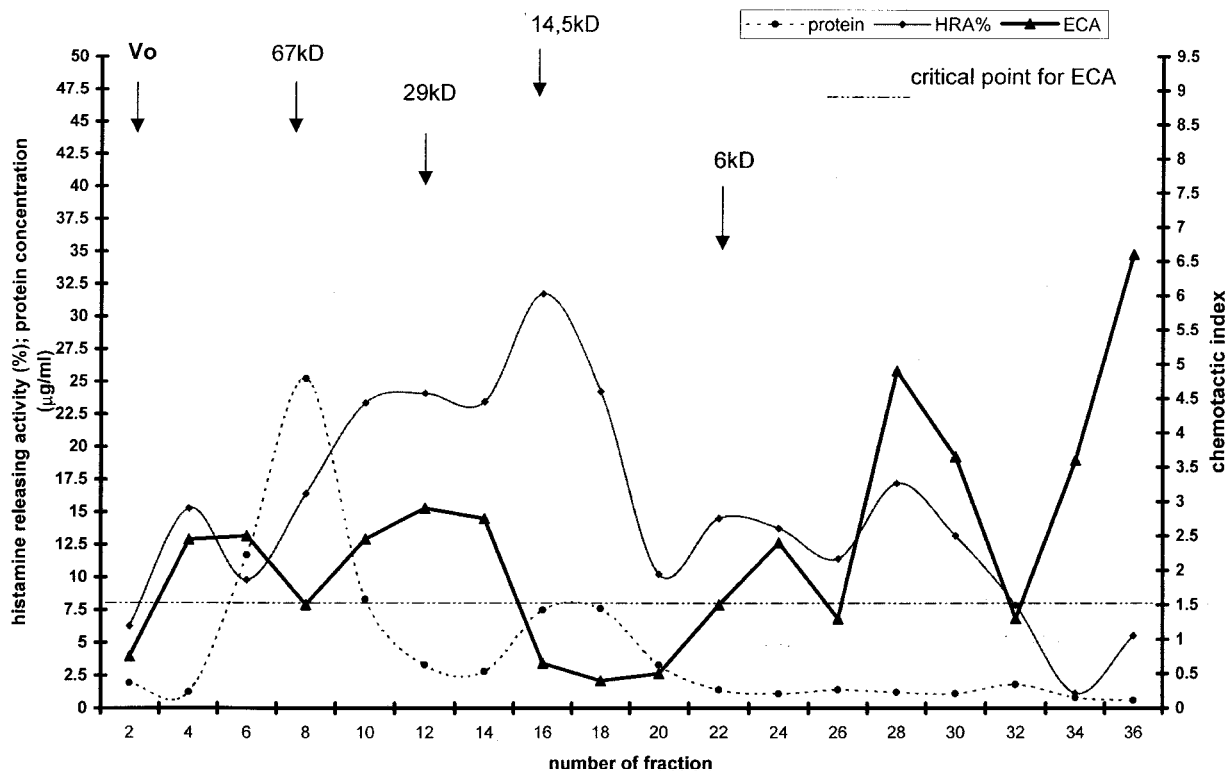

FIG. 3. Gel column chromatography analysis of ECA and HRA generated by mononuclear cells (MNC) from atopic patients (supernatants pooled from three patients).

ECA (+) and ECA (–) non-atopic subjects and stimulation of cells with PHA did not affect NCA. HRA was present in all supernatants from non-atopic subjects (mean 21.34% ± 1.58) although was significantly lower compared with atopic patients (mean 29.52% ± 1.74; $P < 0.05$). After stimulation with PHA, a significant increase in HRA was observed only in ECA (–) subjects (Table 1).

Partial purification of chemotactic activity

Figure 3 shows a typical profile of ECA and HRA along with the total protein concentrations measured in each fraction of pooled supernatants from three atopic asthmatic patients. Eosinophil chemotactic activity (defined as chemotactic index above 1.5) was observed in several fractions. One peak was located above 67 kD; the second about 29 kD but the highest ECA (range 4.9–6.6) was observed in fractions with molecular weight below 6 kD. Significant eosinophil chemokinesis inhibitory activity was detected in fractions with molecular weight between 14.5 and 6 kD. Strong HRA activity (expressed as percentage of histamines released from basophils) was observed in fractions with apparent molecular weight between 67 kD and 14.5 kD.

In pooled MNC supernatants from control subjects (Fig. 4), the first peak of ECA was found in area corresponding to molecular weight above 67 kD. The second peak with significant ECA was present in the area of 14.5 kD and the third peak below 6 kD. Significant eosinophil chemokinesis inhibitory activity was present in fractions with molecular weight around 29 kD. HRA was present in several fractions extending from 67 kD (48.8% histamine release); 29 kD (30.2% histamine release) and 14.5 kD (36.08% histamine release).

Discussion

In this study we demonstrated, that mononuclear cells isolated from patients suffering from atopic asthma and sensitized to house dust mite antigen, as well as from healthy individuals, generate spontaneously ECA. However, atopic and non-atopic subjects demonstrated individual variation in ability to release spontaneously chemotactic activity for eosinophils. In about one-third of atopic patients and in about half of control non-atopic subjects the chemotactic activity was not observed. In fact the supernatants from these ECA (–) subjects had strong blocking effect on chemokinesis of eosinophils. Although the release of ECA by MNC was documented in several studies[27,28] to our knowledge this is the first study reporting the release of inhibitory activity towards eosinophil chemokines.
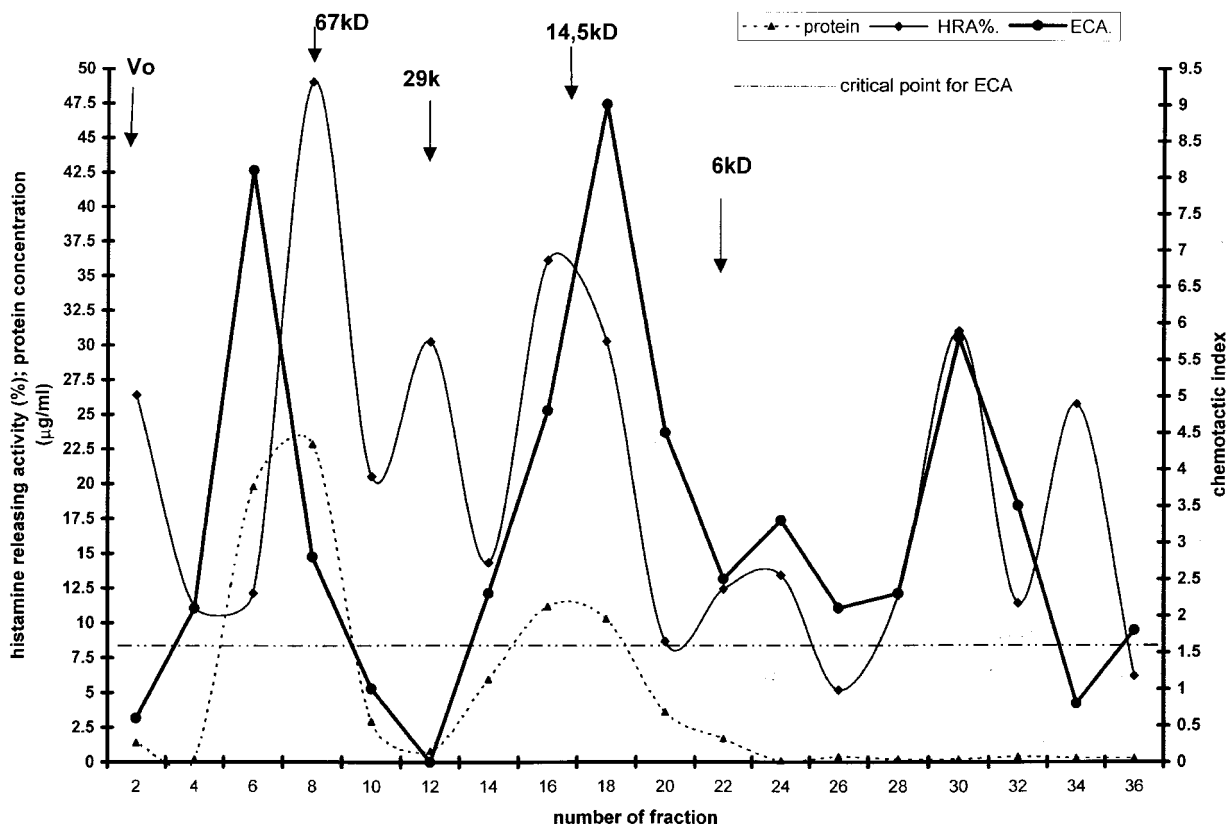

FIG. 4. Gel column chromatography analysis of ECA and HRA generated by mononuclear cells from healthy subjects (supernatants polled from three subjects).

Although the mononuclear cell-derived ECA has not been well characterized it was documented that several cytokines (e.g. IL–5, RANTES or Eotaxin) and non-peptide mediators (e.g. PAF, LTB4) may be involved in this activity.[29] The nature of the eosinophil chemotactic inhibitory activity (ECIA) is more difficult to refer to any specific cytokine or mediator. Gel column chromatography revealed that in atopic patients the major inhibitory activity was present in fractions with molecular weight from 14.5 kD to 6 kD; in non-atopic controls the ECIA was present in fractions around 29 kD. These molecular sizes correspond to the molecular weights of cytokines generated by mononuclear cells. Although to our knowledge no specific cytokine with ECIA has been reported. In addition, in atopic patients, inhibitory activity was also observed in low-molecular-weight fractions. This activity may correspond to non-peptide mediators released by MNC, e.g. eicosanoids or histamines released from basophils, which could contaminate MNC preparations. Accordingly, we have recently reported inhibition of eosinophil chemotaxis to RANTES after incubation of eosinophils with histamine.[30] Although to our knowledge the presence of eosinophil chemokinesis inhibitory activity has not yet been reported, a generation of cytokines with reciprocal biological effects has been previously described. Alam *et al.*[31] described a release by MNC from asthmatic patients with both histamine releasing activity and histamine release inhibitory activity. These activities were subsequently identified as specific cytokines; HRA consisted mostly of MCP–1 and RANTES, while IL–8 was considered to be responsible for histamine release inhibitory activity.

In our study gel chromatography revealed that MNC supernatants contained both chemotactic and eosinophil chemokinesis inhibitory activity in different fractions. Furthermore, stimulation of ECA 'non-releasing' mononuclear cells in either a specific (antigen) or non-specific manner (PHA) resulted in the appearance of ECA with magnitude similar to that observed in spontaneous ECA (+) supernatants. It may suggest that cell activation results in generation (release) of chemotactic molecules, that overweigh the effect of inhibitory molecules. Alternatively, it is possible that the activation of mononuclear cells may decrease the release of ECIA, thus allowing for unopposed activity of molecules with chemotactic activity.

When we analysed neutrophil chemotactic and histamines release activity in subgroups of patients, no differences in the spontaneous generation of NCA and HRA were observed between ECA (+) and ECA (–) subjects, indicating that the heterogeneity of the cytokines release could be selective, being restricted only to ECA. However, HRA could be differentially modulated by non-specific stimulus (PHA) in subgroups non-atopic patients, with only ECA (–) sub-

jects demonstrating an increase in HRA. Gel column chromatography analysis showed heterogeneity of molecular weight for all examined activities (ECA, NCA and HRA) indicating multifactoral nature of relatively simple *in vitro* cellular responses.

It has been documented that the activation of eosinophils with the cytokines, which are synthesized and excreted, by mononuclear cells may be vital for the development of allergic inflammation in the respiratory tract.[31] The immigration of eosinophils into the tissues is conditioned by the presence of Th2-cell-derived IL–5, to which a significant role in maturing and activation of eosinophils is attributed.[10] Other molecules, which activate eosinophils and basophils and have chemotactic activity for these cells include RANTES and other CC chemokines: MCP–1, MCP–2, MCP–3, MCP–4, Eotaxin, MIP–1α, MIP–1β, MIP–1γ.[14,17] Our study was not aimed at determining which of the known cytokine is responsible for the eosinophil signalling. We can speculate however, that ECA measured in chemotactic assay results from the coordinated activity of a number of chemotactic cytokines with both pro- and anti-chemotactic activity. Our results emphasize a complicated nature of allergic inflammation, which may not be visible, when a single mediator or cellular component is studied. These data also stress the relevance of studying biological activities of crude cells/tissues products in addition to analysis of generation of well-defined mediators. Such an approach may allow for the better understanding of complex relations, between the different mediators present in the biological system, where the final biological effect of a single mediator (cytokine) depends on its interactions with other factors.

In conclusion we demonstrated that mononuclear cells isolated from the peripheral blood of patients with atopic asthma and from healthy individuals generate *in vitro*, not only eosinophil chemotactic activity, but also eosinophil chemokinesis inhibitory activity. MNC of subjects generating eosinophil chemokinesis inhibitory activity were capable of producing ECA after stimulation with antigen or PHA in atopic and control subjects respectively.

Acknowledgement

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