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Blood eosinophil leukotriene C_4 production in asthma of different severities

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Blood eosinophil leukotriene C_4 production in asthma of different severities. M. Laviolette, C. Ferland, J-F. Comtois, K. Champagne, M. Bossé, L-P. Boulet. ©ERS Journals Ltd 1995.

ABSTRACT: In asthma, activation and recruitment of eosinophils to the bronchial mucosa amplifies many cellular functions. The blood eosinophil count and the number of hypodense eosinophils increase with asthma severity. Eosinophils produce numerous proinflammatory mediators in response to a variety of agonists, notably the peptido-leukotriene (LT) C_4, a potent bronchoconstrictor.

In this study, we have evaluated blood eosinophil LTC_4 release and its modulation by cytokines in normal individuals and in subjects with asthma of various severities: mild (β_2-agonist on demand); moderate (inhaled steroids on a regular basis); and severe (inhaled and oral steroids on a regular basis). Eosinophils were isolated using a modified Percoll gradient technique, which recovers both hypodense and normodense eosinophils in a global cell population.

Eosinophils released detectable amounts of LTC_4 only in the presence of the stimulus (calcium ionophore A23187, 2 μM). The ionophore-induced LTC_4 release was greater in moderate asthmatics (mean±SEM 5.7±1.3 pg×10^3/250,000 eosinophils) than in normal individuals (1.6±0.4 pg×10^3/250,000 eosinophils), mild asthmatics (1.8±0.3 pg×10^3/250,000 eosinophils) and severe asthmatics (2.0±0.3 pg×10^3/250,000 eosinophils). Granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-5 (IL-5) amplified the ionophore-induced LTC_4 release in the four groups from 1.9 to 2.6 and 1.9 to 2.8 fold, respectively. Interleukin-3 (IL-3) did not increase LTC_4 production except by the eosinophils of the severe asthmatics whose ionophore-induced LTC_4 production was enhanced by 1.9 fold.

These data demonstrate that the asthmatic bronchial inflammatory process may modify blood eosinophil LTC_4 release and its modulation by cytokines according to asthma severity and treatment.

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Asthma is an airway inflammatory disease characterized by a mucosal eosinophil infiltration [1]. These cells are believed to be recruited from bone marrow and circulating blood to the bronchi by cytokines and chemotactic factors released in the inflamed bronchial tissue [2, 3]. Cytokines, notably granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and interleukin-5 (IL-5) modulate eosinophil functions: they enhance eosinophil *in vitro* survival, cytotoxicity, phagocytosis, and granule and mediator release in response to stimuli [4–8]. GM-CSF, IL-3 and IL-5 have been detected in the blood of individuals with allergic asthma, and it has been shown that the lymphocytes of asthmatics can secrete these cytokines spontaneously, as well as after activation [9, 10]. Blood eosinophil count and the number of hypodense eosinophils, which are considered as activated or immature cells, increase with the severity of asthma [2, 11, 12].

Eosinophils produce many mediators, notably the peptido-leukotriene (LT) C_4, a powerful bronchoconstrictor, which also increases vascular permeability and mucus secretion [13]. Up to now, in patients with asthma, studies have evaluated the release of LTC_4 by blood eosinophil subpopulations; and have reported that, upon stimulation with calcium ionophore A23187, hypodense eosinophils release either less [14] or more [15–19] LTC_4 than normodense eosinophils. These conflicting results may be explained, at least in part, by the heterogeneity in asthma severity, the diversity in the eosinophil density ranges considered as normodense and hypodense from one study to another, and by the differences in techniques used for the purification of eosinophils. We recently described a modified eosinophil isolation technique that recovers most eosinophils, normodense and hypodense, from blood cell preparations [20]. We believe that the resulting eosinophil preparation is representative of the global eosinophil population and can be used to compare different groups of subjects on the basis of a whole eosinophil population instead of on selected subpopulations.

In this study, we used this eosinophil isolation technique to evaluate the LTC_4 production of blood eosinophils from normal and asthmatic subjects with various degrees of asthma in response to ionophore A23187, and its modulation by the cytokines GM-CSF, IL-3 and IL-5. Our

data showed that eosinophils of subjects with moderate asthma presented an increased capacity to produce LTC_4 compared to normal individuals and subjects with mild and severe asthma. GM-CSF and IL-5 upregulated ionophore A23187-induced LTC_4 production in all groups of subjects, and IL-3 did so in severe asthmatic eosinophils only.

Materials and methods

Evaluation of subjects

Twenty three subjects were recruited into the study, and all were nonsmokers. Five were normal subjects without history of allergy or asthma and eighteen met the criteria of the American Thoracic Society for the diagnosis of asthma [21]. In this study, the severity of asthma was determined by the medication required to achieve control of asthma symptoms, according to the International Consensus Report on Treatment of Asthma [22]. Mild asthmatics were defined as patients with a morning prebronchodilator forced expiratory volume in one second (FEV1) >80% of the predicted value, and requiring β_2-agonist on demand less than three time a week. Moderate asthmatics were defined as patients requiring at least 2 inhalations of β_2-agonist twice a day and inhaled steroids (beclomethasone dipropionate 1,000 µg or budesonide 800 µg *q.d.*) to control asthma and achieve a FEV1 >80% of the predicted value. Severe asthmatics were defined as patients requiring β_2-agonist regularly, in addition to beclomethasone dipropionate 2,000 µg or budesonide 1,600 µg *q.d.* and oral steroid (prednisone) ≥10 mg *q.d.* to control their asthma symptoms.

The inclusion criteria were: stable asthma symptoms and treatment for more than 3 months, according to the International Consensus Report on Treatment of Asthma [22], no need of oral steroid over the last year preceding the study except for severe asthmatics, no need of inhaled steroid over the 3 months preceding the study in the mild asthmatic group, no history of allergy except allergic asthma, no use of other drugs and no disease other than asthma. All subjects gave an informed consent to the study, which was approved by the local Ethics Committee.

The subjects underwent measurement of forced expiratory flows and nonallergic airway responsiveness, and blood sampling. Forced expiratory flows were measured in the morning after at least 8 h without β_2-agonist on a PFT II Vitalograph Spirometer, and were expressed as percentage of predicted values [23]. Airway response to methacholine was measured according to the method described by JUNIPER *et al.* [24], and was expressed as the concentration provoking a 20% fall in FEV1 (PC20).

Blood cell processing and eosinophil purification

Venous blood (120 mL) was collected early in the morning in ethylenediamine tetraacetic acid (EDTA) tubes. The blood was centrifuged to remove the plasma, and the cell pellet was sedimented on Dextran (Pharmacia LKB; Uppsala, Sweden). Leucocytes were centrifuged on Ficoll-Paque (Pharmacia) for 20 min at 700×g. The cell pellet containing the granulocytes was resuspended and red cells lysed with distilled water. Eosinophils were isolated using the technique described by KOENDERMAN *et al.* [25], and modified in our laboratory to increase eosinophil recovery and to avoid discarding eosinophil subpopulations [20].

Briefly, granulocytes (75×10^6 cells) in one millilitre of Ca^{++}/Mg^{++}-free Hank's balanced salt solution (HBSS) (Gibco BRL; Grand Island, NY, USA) with 5% foetal bovine serum (FBS) (Gibco) were incubated with 10^{-8} M N-formyl-methionyl-phenylalanine (fMLP) (Sigma, St-Louis, MO, USA) for 10 min at 37°C. The cells were then suspended in HBSS (Gibco) and overlayed on a two-step Percoll (Pharmacia LKB; Uppsala, Sweden) gradient with densities of 1.078 and 1.100 $g\cdot mL^{-1}$, respectively. After centrifugation, cells at the interfaces were recovered, washed and counted. Eosinophil purity and recovery were assessed on each cellular fraction after Percoll (Pharmacia) separation. Total cell counts were determined using a hemocytometer, and differential cell counts were performed using the technique described previously [26]. Cell staining was carried out with Diff-Quik stain (Baxter Canlab Division; Mississauga, Ontario, Canada) and at least 400 cells were counted. Cell viability, estimated by trypan blue exclusion, was always greater than 98%. Eosinophil recovery was expressed as the percentage of eosinophils present at the interface 1.078–1.100 $g\cdot mL^{-1}$ on Percoll gradient over the total number of eosinophils present at all interfaces and in the cell pellet.

Eosinophil stimulation and LTC_4 measurement

Eosinophils isolated at the interface 1.078–1.100 $g\cdot mL^{-1}$ on Percoll gradient were suspended in RPMI (Gibco) containing $MgCl_2$ (0.5 mM), $CaCl_2$ (2 mM), FBS (Gibco) 10%, and 1% penicillin-streptomycin (Gibco). Eosinophils from subjects with moderate or severe asthma were incubated either with various concentrations of GM-CSF, IL-3 and IL-5 (Genzynes; Cambridge, MA, USA) for 30 min, or with GM-CSF (100 $U\cdot mL^{-1}$), IL-5 (50 $ng\cdot mL^{-1}$) and IL-3 (50 $U\cdot mL^{-1}$) for variable periods of time (15–360 min). These experiments were performed to determine whether the dose-response and the kinetics of the cytokine modulation of the LTC_4 production by eosinophils of subjects with moderate and severe asthma were similar to those observed in eosinophils of normal individuals and subjects with mild asthma or allergic rhinitis [20]. The cells were then stimulated for 15 min with 2 µM calcium ionophore A23187 in microtubes. It had previously been found that, in normal individuals and subjects with mild asthma, this dose of ionophore was effective without affecting cell viability, and that LTC_4 production plateaued after 5 min of incubation with ionophore [19].

A second set of experiments evaluated whether the LTC_4 production of eosinophils obtained from the different groups was maximal or suboptimal in incubation with 2 µM calcium ionophore. Cells from subjects of

Table 1. – Subjects' clinical and physiological parameters

Group	Medication	Sex F/M	Age* yrs	Blood eosinophil* cells×10^9·L^{-1}	PC_{20}** mg·mL^{-1}	FEV_1* % pred
Normal	Nil	3/2	35±5	0.1±01 (≤0.1)	121.9±1.8	98±5
Asthma						
Mild	Inhaled β_2-agonists+	3/3	25±3	0.37±0.06 (0.1–0.5)	1.2±1.7	88±6
Moderate	Inhaled steroids++	6/0	35±5	0.35±0.07 (0.2–0.6)	0.8±1.6	87±5
Severe	Inhaled & oral steroids+++	1/5	46±3	0.48±0.23 (0.01–1.2)	ND	65±5

*: mean±SEM and range in parenthesis. **: geometric mean±SEM, values >254 mg·mL^{-1} were considered as equal to 254 mg·mL^{-1}. +: ≤3 times a week; ++: beclomethasone 1,000 µg or budesonide 800 µg daily, and inhaled β_2-agonists less than twice daily; +++: beclomethasone 2,000 µg or budesonide 1,600 µg daily, oral steroids 10–30 mg daily, and inhaled β_2-agonists 2–8 puffs daily. ND: not determined; F: female; M: male; FEV_1: forced expiratory volume in one second; PC_{20}: concentration provoking a 20% fall in FEV_1.

each group were stimulated with increasing doses of ionophore from 1–20 µM or dimethyl sulphoxide (DMSO) (Sigma) used as vehicle for 15 min. Thereafter, purified blood eosinophils obtained from the different groups were compared for their ionophore-induced LTC_4 production, and its modulation by the cytokines studied, using the suboptimal dose of ionophore determined and optimal incubation conditions with cytokines. At the end of all experiments, the cell suspensions were centrifuged and the supernatants kept at -70°C for LTC_4 determination. LTC_4 was measured using enzyme immunoassay kits according to the manufacturer's instructions with a detection limit of 10 pg·mL^{-1} (Cayman Chemical Co., Ann Arbor, MI, USA).

Statistical analysis

Results are expressed as mean±SEM, as data were normally distributed. Efficacy of Percoll gradients in the purification and recovery of blood eosinophils were analysed using a one-way analysis of variance (ANOVA). A similar analysis was performed for LTC_4 production. As a significant overall difference was observed, pairwise differences between mean groups were evaluated using Scheffé's comparison. To analyse the effects of cytokines on eosinophils in each group, we performed a randomized block design where subjects represent blocks. When a significant overall difference was observed, each cytokine mean result was compared with that of the diluent. The Dunnett's procedure was used for making these comparisons. The statistical analysis system (SAS) was used for all analysis.

Results

Subjects' characteristics

The subjects' characteristics are presented in table 1. Mean FEV_1 of subjects with mild and moderate asthma were similar to that of normal subjects. Patients with severe asthma had a significantly lower baseline FEV_1 (65±5% of predicted) (p=0.002). The PC_{20} methacholine was >16 mg·mL^{-1} in normal subjects, except for one who had a PC_{20} limit of 11.2 mg·mL^{-1} without any symptoms of asthma. PC_{20} methacholine was lower in mild and moderate asthmatics (geometric mean 1.2±1.7 and 0.8±1.6 mg·mL^{-1}, respectively). PC_{20} could not be measured in severe asthmatics due to the severity of their asthma. The total number of peripheral blood eosinophils was increased in patients with asthma (≥0.3×10^9 cells·L^{-1}) in comparison with normal subjects (0.1±0×10^9 cells·L^{-1}).

Eosinophil recovery and purity on Percoll gradients in each group

Figure 1 shows the purity and recovery of blood eosinophils harvested from the Percoll gradients in each group. The mean purity and recovery were similar between each group (p=0.32 and p=0.76, respectively; multiple comparison p=0.59). The mean purity varied from 85±6% in moderate asthmatics to 95±2% in severe asthmatics. Contaminant cells were neutrophils. The mean recovery varied from 70±9% in moderate asthmatics to 84±5% in normal subjects.

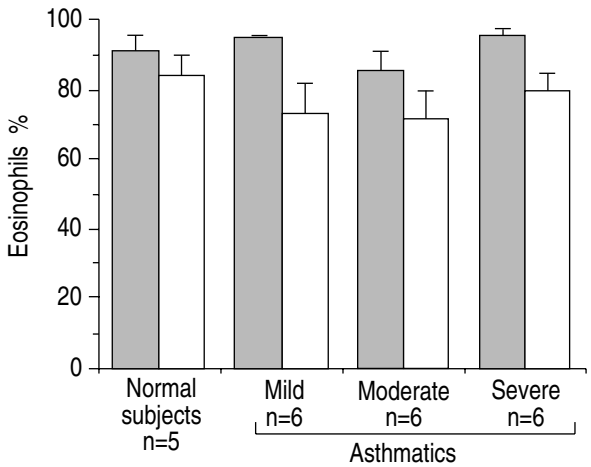

Fig. 1. – Efficacy of Percoll gradients for the isolation of blood eosinophils. The mean purities (▭) were similar (>85%) in the four groups. The mean recoveries (▭) tended to be lower in asthmatics but still remained ≥70%.

Dose-response and kinetics of eosinophil LTC_4 production modulation by GM-CSF, IL-5 and IL-3

Figure 2a and b show the cytokine dose-response and kinetics experiments, respectively, in which the eosinophils of asthmatics were incubated with various concentrations of cytokine for 30 min or a fixed dose of cytokine for 15–360 min before stimulation with ionophore 2 μM. Cytokines did not induce any detectable release of LTC_4 in the absence of ionophore. The dose-response experiments indicated that enhancement of ionophore-induced LTC_4 production by GM-CSF, IL-5 and IL-3 preincubation was dose-dependent. Optimal concentrations used for eosinophil induction were: GM-CSF 100 U·mL^{-1}, IL-5 100 ng·mL^{-1} and IL-3 100 U·mL^{-1}, with no further increase at higher cytokine concentrations. These cytokine concentrations were used for the subsequent experiments, except for IL-5 and IL-3, where a concentration of 50 ng·mL^{-1} and 50 U·mL^{-1} were preferred due to the cost of the cytokine. The kinetics of the cytokine modulation of LTC_4 production showed that the enhancement of ionophore-induced LTC_4 generation was optimal at 30 min for GM-CSF and IL-5, and at 6 h for IL-3. Note, however, that IL-3 probably did not reach a plateau response at 6 h.

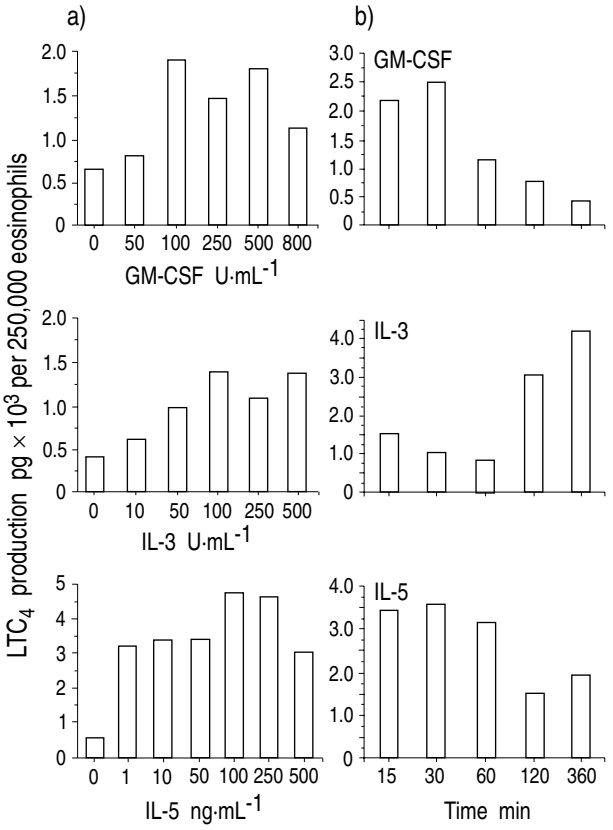

Fig. 2. – a) Dose-response and b) kinetics of eosinophil ionophore-induced LTC_4 production modulation by cytokines. Eosinophils from moderate and severe asthmatic subjects were incubated: a) with various concentrations of GM-CSF, IL-3 and IL-5 for 30 min; and b) with GM-CSF (100 U·mL^{-1}), IL-5 (50 ng·mL^{-1}) and IL-3 (50 U·mL^{-1}) for variable periods (15–360 min). (Eosinophils from one subject for each set of experiments). LTC_4: leukotriene C_4; GM-CSF: granulocyte/macrophage colony-stimulating factor; IL-3: interleukin-3; IL-5: interleukin-5.

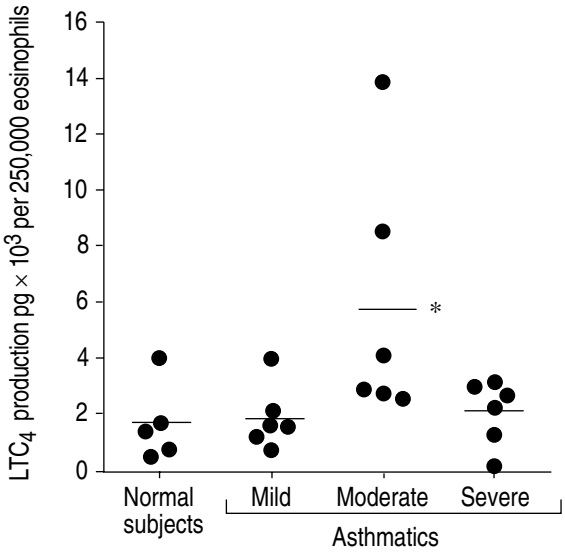

Fig. 3. – Comparison of the eosinophil LTC_4 production of the different groups. Eosinophils did not spontaneously release detectable amount of LTC_4. Eosinophils of moderate asthmatics stimulated with ionophore A23187 (2 μM) for 15 min released more LTC_4 than those of normal individuals, and of mild and severe asthmatics ($p<0.05$). LTC_4: leukotriene C_4.

LTC_4 production by purified blood eosinophils from the different groups

The eosinophils released no detectable LTC_4 spontaneously. As reported previously [19], the ionophore dose of 2 μM was effective and suboptimal in all types of subject, so that this dose was chosen for the subsequent experiments to compare the LTC_4 production of eosinophils from each subject of each group. In these experiments, upon stimulation with ionophore (2 μM) for 15 min, eosinophils of moderate asthmatics released ≥2.8 fold more LTC_4 than those of normals, and of mild and severe asthmatics ($p<0.05$) (fig. 3). The ionophore-induced LTC_4 productions were similar in the eosinophils of normal individuals and of mild and severe asthmatics.

Effects of cytokines on eosinophil LTC_4 production in each group of subjects

Eosinophils of each subjects were incubated with one of the cytokines studied, GM-CSF (100 U·mL^{-1}, 30 min), IL-3 (50 U·mL^{-1}, 6 h) or IL-5 (50 ng·mL^{-1}, 30 min) before stimulation with ionophore A23187. GM-CSF and IL-5 but not IL-3 upregulated the ionophore-induced LTC_4 release (≥2.0 fold; $p<0.05$) in normals and mild asthmatics (fig. 4a and b). In moderate asthmatics, the cytokines GM-CSF and IL-5 also amplified the response to ionophore by a similar magnitude (1.9 fold) ($p<0.05$), even though the ionophore-induced LTC_4 production of this group was already increased in comparison to the other groups (fig. 4c). IL-3 slightly increased the LTC_4 release by the eosinophils of the moderate asthmatics but this effect was not significant. In severe asthmatics, the three cytokines studied, GM-CSF, IL-5 and IL-3, upregulated the ionophore-induced LTC_4 production by ≥1.9 fold (fig. 4d; $p<0.05$).

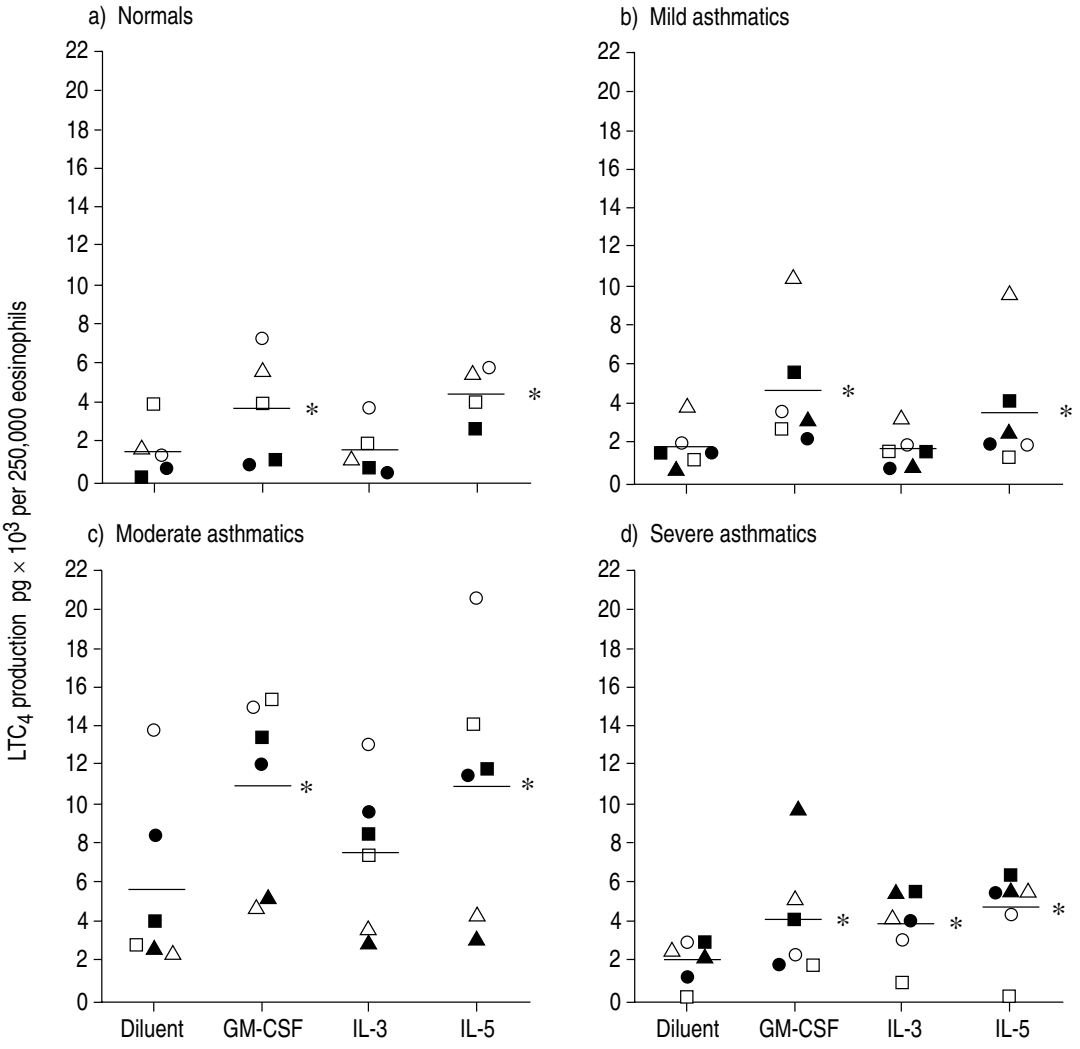

Fig. 4. – Effects of cytokines on eosinophil ionophore-induced LTC_4 production in: a) normal individuals; b) mild asthmatics; c) moderate asthmatics; and d) severe asthmatics. Each symbol represents one subject. Eosinophils were incubated with or without ionophore A23187 (2 μM) after preincubation with the diluent or a cytokine. Cytokines did not induce any detectable release of LTC_4 in the absence of stimulus. GM-CSF and IL-5 but not IL-3 provoked a significant increase of ionophore-induced LTC_4 release in normal individuals and mild and moderate asthmatics (∗: $p<0.05$). In severe asthmatics, the three cytokines enhanced the ionophore-induced LTC_4 production (*: $p<0.05$). For abbreviations see legend to figure 2. Horizontal bar: mean.

Discussion

Eosinophils are considered as important effector cells in the pathogenesis of asthma [3, 27]. These cells release numerous potent mediators, notably LTC_4, formerly known as the slow-reacting substance of anaphylaxis. The increased blood eosinophil count and number of hypodense eosinophils observed suggest that blood eosinophils are activated in asthma [2]. So far, studies have focused on the LTC_4 production by eosinophil subpopulations of normal or low density [14–19]. We have developed a purification technique that recovers both normal and low density eosinophils in a global population [20]. In this study, we used this technique to compare blood eosinophil populations obtained from normal individuals and from subjects with various degrees of asthma. It shows that asthma, depending on its severity, activates blood eosinophils to release more LTC_4 *in vitro* upon stimulation and alters their response to cytokines.

In the mild asthmatic's group, the eosinophil responses to ionophore and cytokines in terms of LTC_4 production were similar to those observed in normals. These data do not mean that the eosinophils of mild asthmatics were not activated in any other function. Indeed, bronchial inflammatory processes have been found in subjects with mild asthma and their blood eosinophil counts are increased, indicating that eosinophils are recruited into the airway in mild asthma [28–30]. However, our data suggest that, in these subjects, the blood eosinophil activation process is not important enough to alter the ionophore-induced LTC_4 production. In contrast, moderate asthmatics presented an increase in eosinophil ionophore A23187-induced LTC_4 release. Moderate asthma causes a more intense eosinophilic bronchial inflammation than mild asthma [27]. This increased airway inflammation is likely to induce a greater degree of blood eosinophil activation and recruitment to the bronchial mucosa, and to increase the eosinophil capacity to produce LTC_4.

The moderate asthma group differed from the mild asthmatic and normal groups by the use of inhaled steroids, which are required to achieve asthma control. At the dose used, some systemic effects could occur, but are not likely to be significant [31, 32]. In a double-blind, cross-over study, we recently evaluated the effect of the addition of inhaled steroids (beclomethasone dry powder, 800 µg *q.d.*) for a period of 6 weeks to the treatment of subjects with asthma which required more than two inhalations of β_2-agonist *b.i.d.* to assure adequate symptom control. The addition of inhaled steroid led to better control of their asthma and to a decrease in blood eosinophil count, LTC_4 production, and GM-CSF *in vitro* priming compared to a placebo [33]. We postulated that this effect was due to a reduced bronchial inflammation and release of proinflammatory mediators acting on blood eosinophils.

Recently, inhaled steroids have been shown to reduce the number of blood hypodense eosinophils, and the authors also postulated that this reduction was mediated mostly through the topical effect of inhaled steroids [34]. In comparison to mild asthmatics, moderate asthmatics were also using β_2-agonists regularly, which could hypothetically alter LTC_4 production. We recently found that regular use of salmeterol, a long-acting β_2-agonist, *b.i.d.* for 2 months did not modify LTC_4 production of blood eosinophils [35]. Therefore, based on these observations and since the subjects did not use other medication, we believe that the increased blood eosinophil LTC_4 production seen in the moderate asthmatics is not due to the difference in the medication used to control asthma symptoms but to the severity of asthma.

The enhanced eosinophil LTC_4 production observed in the moderate asthmatics is most likely to be due to an increased activity of signalling cellular pathways upregulating the activity of the enzymes involved in the synthesis of LT: phospholipase A_2, 5-lipoxygenase and/or five-lipoxygenase activating protein [13, 36–38]. Since, as described previously, GM-CSF and IL-5 enhance LTC_4 production by eosinophils of normals and asthmatics, we may postulate that the eosinophils of moderate asthmatics have been primed *in vivo* by these cytokines to produce more LTC_4 [39, 40]. Interestingly, the eosinophils of the moderate asthmatics showed the *in vitro* enhancement of cytokine-induced LTC_4 production even though they spontaneously presented an increased ionophore-induced LTC_4 production compared to normals and mild asthmatics. The explanation for these observations remains speculative and includes *in vivo* priming by another mechanisms and/or cytokines.

Eosinophils of the severe asthma group had LTC_4 production similar to normal cells in response to ionophore stimulation. The fact that these subjects had clinically severe asthma and increased blood eosinophil counts indicated that they had more intense airway inflammation. Consequently, their blood eosinophils should have presented a spontaneously amplified ionophore-induced LTC_4 production, as seen in moderate asthmatics. However, to control their asthma symptoms, these subjects took high doses of inhaled steroids and oral steroids. As mentioned above, low doses of inhaled steroids decreased blood eosinophil LTC_4 production of asthmatics with symptomatic asthma [33]. Therefore, the combined high dose inhaled steroids and oral steroids probably modulated bronchial cytokine production more efficiently, and also acted directly on blood eosinophils following their systemic absorption, inhibiting, at least partly, their activation and normalizing the ionophore-induced LTC_4 release [41–43]. Interestingly, the steroids did not inhibit the cytokine-induced enhancement of ionophore-induced LTC_4 production. These data further support the hypothesis that the increase in ionophore-induced LTC_4 release observed in moderate asthmatics, and the GM-CSF and IL-5 enhancement of the ionophore-induced LTC_4 production observed in all groups are mediated through independent mechanisms. Indeed, in monocytes, the phospholipase activation involves at least two different mechanisms: a direct calcium-dependent and a protein kinase-dependent activation [44].

The cytokine IL-3 significantly enhanced the ionophore-induced LTC_4 release only in the severe asthmatic group. These data support the hypothesis that the effects of IL-3 are mediated through different cellular mechanisms than those involving IL-5 and GM-CSF, and suggest that its effect on eosinophils is related to the intensity of the asthmatic inflammatory process or the effect of systemic steroid treatment on eosinophils [45]. The modulation of phospholipase activity by cytokines may be due to a protein kinase-dependent signal. The implication of different kinases as modulators of phospholipase activity, such as protein kinase C (PKC) and Janus kinase (JAK) kinase involved in IL-3 and GM-CSF signal transduction, remains to be investigated [45].

The technique used to isolate blood eosinophils provides a high eosinophil recovery (≥70%) and purity (≥90%) rates, and we believe that the resulting eosinophil populations are representative of the total blood eosinophil populations and adequate for comparison between normal individuals and asthmatics. In this technique, granulocytes were incubated for 10 min with a low concentration of fMLP (10^{-8} M) to decrease neutrophil density below 1.078 $g \cdot mL^{-1}$ and, consequently, to purify eosinophils from neutrophils. We previously showed that such a short incubation with a low dose of fMLP did not activate eosinophils of normal individuals, and mild and moderate asthmatics to release more LTC_4 on stimulation with ionophore nor modify their *in vitro* response to cytokines [20]. Moreover, eosinophils isolated by this technique released similar amounts of LTC_4 to eosinophils isolated by immunomagnetic selection using anti-CD16 monoclonal antibodies [20]. Therefore, we believe that the differences seen in eosinophil LTC_4 production between the various groups of subjects are very unlikely to be due to the eosinophil isolation technique or cell incubation procedures.

We also took care to avoid any concomitant disease, which could provoke eosinophil activation and recruitment, such as allergic rhinitis, drug allergy, allergic dermatitis and parasitic infestations, and which could add to the effect of the asthmatic bronchial inflammatory process on blood eosinophils.

The increase in asthma symptoms and in airway

responsiveness are very likely to be associated with an increase in bronchial inflammation. Therefore, we assume that a more intense asthmatic bronchial inflammatory process increases the release of chemotactic factors and cytokines by cells present in the bronchial wall, and, consequently, increases the recruitment and the activation of blood eosinophils. Eosinophils are mostly tissue cells, and the degree of blood eosinophil activation should reflect the intensity of the tissue inflammation. This study shows that, indeed, the eosinophils of asthmatics are activated and that the degree of activation varies with the asthma severity and also with the use of systemic treatment. However, cellular mechanisms and mediators which mediate these effects are not clearly defined.

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