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Increased generation of the arachidonic metabolites LTB_4 and 5-HETE by human alveolar macrophages in patients with asthma: effect *in vitro* of nedocromil sodium

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Increased generation of the arachidonic metabolites LTB_4 and 5-HETE by human alveolar macrophages in patients with asthma: effect in vitro of nedocromil sodium. M. Damon, C. Chavis, J.P. Daures, A. Crastes de Paulet, F.B. Michel, Ph. Godard.

ABSTRACT: Alveolar macrophages (AM) are the principal resident phagocytes in the human lung, and play a major role in local defence against environmental agents. It is now known that during asthma these cells take part in the amplification of the inflammatory mechanism. It has been demonstrated *in vitro* that they can be activated to generate leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE), mediators with potent pharmacological properties. These two arachidonic metabolites were identified and quantified by reversed phase high performance liquid chromatography (HPLC) performed in cell suspensions, and in cell free supernatants. AM from asthmatics, after stimulation by the calcium ionophore A23187 or opsonized zymosan, released significantly ($p<0.05$) more LTB_4 than those from healthy subjects. The increase in LTB_4 release could be evidence for *in vivo* activation. On the other hand, the levels of 5-HETE in the AM from asthmatics were significantly ($p<0.03$) higher than those in cells from healthy subjects. This intracellular increase could be correlated with a greater migratory ability of these inflammatory macrophages, as observed for eosinophils. The clinical efficacy of nedocromil sodium may be partly related to the decreases in LTB_4 releasability and intracellular 5-HETE levels observed only in AM from asthmatic patients.

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Bronchial asthma defined as a reversible airway obstruction, is mainly characterized by the association of smooth muscle spasm with an airway inflammation [1]. Some evidence suggests that bronchial hyperreactivity, a main feature of asthma, is caused by airway inflammation [2]. Bronchial inflammatory cells, and the mediators they release, play a central role [3] in the pathophysiology of asthma. Thus, cells of the bronchoalveolar lumen are an important subject of study because of their direct exposure to allergens and irritants and their participation in the mechanism of reversible airway obstruction observed in allergic bronchial asthma.

These cells can be obtained by bronchoalveolar lavage (BAL) [4] and studied *in vitro*, either under resting conditions or after specific stimulations. Besides these cells, alveolar macrophages (AM) are the principal resident phagocytes in the human lung and play a major role in local defence. It is known that AM take part in the amplification of the inflammatory mechanism during asthma [5]. As demonstrated *in vitro*, AM can be activated to release inflammatory mediators from membrane phospholipids including paf-acether [6] and arachidonic acid metabolites [7–11]. These mediators have potent pharmacological properties [12–14]; some of the arachidonic acid metabolites are able to recruit other cells [15–17] and participate in the imunological mechanism of asthma [18, 19]; others have bronchoconstrictor effects (components of SRS-A [20]) or can induce the release of histamine [21]. In previous work we have found that AM can release prostaglandins and thromboxane B_2 (TxB_2) [7], and also 5- lipoxygenase metabolites such as leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid 5-HETE), when they were stimulated with a calcium ionophore and opsonized zymosan [9, 22], and can release leukotriene D_4 (LTD_4) when they are subcultured in the presence of arachidonic acid for 6–24 h [11, 23]; an increase in the mediator releasability could be the result of the *in vivo* activation of AM, as it was suggested by CLUZEL *et al.* [24], who showed that the capacity of AM to release oxygen species was correlated with the severity of the asthma.

Nedocromil sodium is the disodium salt of a pyranoquinoline dicarboxylic acid (4,6-dioxo-9-ethyl-10-propyl-4H6H-pyrano-3,2-9-quinoline-2,8-dicarboxylic acid). It has been shown to be effective in inhibiting immediate and late phase reaction in response to antigen challenge [25, 26], to exercise [27], and to SO_2 challenges [28]. It also reduces bronchial reactivity in

pollen-sensitive individuals during the pollen season [29]. The aim of the present work was to determine its effects on LTB_4 and 5-HETE synthesis and releasability in AM from asthmatic and normal subjects.

Patients and methods

Description of subjects

The study included 11 healthy volunteers (HS) ranging in age from 22–60 yrs (mean±SD=40±15), and 12 asthmatic patients (AA) ranging in age from 16–45 yrs (32±12). All were nonsmokers. Asthma was diagnosed according to the ATS statement [30]; a reversible airway obstruction of at least 15% predicted after beta-agonist spray was required during the last year. Allergy was assessed by the presence of at least three positive skin tests with common aero-allergens and a high immunoglobulin E (IgE) level in the blood. None of the healthy subjects or patients were on medication; theophylline and beta-agonists were stopped 2 days prior to the study; disodium cromoglycate and inhaled corticosteroids had been discontinued for at least 15 days; ketotifen had been discontinued for three weeks. The effect of nedocromil sodium was observed *in vitro* on AM from only 9 HS and 10 AA.

Bronchoalveolar lavage (BAL)

Bronchoscopy was performed with a flexible fibreoptic instrument (Olympus BF-83) 15 min after premedication with atropine (0.25 mg), metopimazine (10 mg) and local anaesthesia with 5–10 ml of 1% lidocaine. BAL was performed by instillation of 300 or 400 ml of 0.9% saline at room temperature into a subsegmental bronchus of the right middle lobe, followed by gentle aspiration, as previously described [3, 4].

Materials

Leukotriene B_4 and 5-hydroxyeicosatetraenoic acid (5-HETE) were purchased from Bioart (Meudon, France). Zymosan, ionophore A23187, and nor-dihydroguaîaretic acid (NDGA) came from Sigma Chemical Co (St. Louis, Missouri). Petri dishes for cell cultures were from Becton Dickinson (Grenoble, France), and media (medium 199 and heat-inactivated foetal calf serum, FCS) from Flow Laboratories (Putaux, France). High perfromance liquid chromatography (HPLC)-grade solvents were purchased from SDS (13124 Peypin, France).

Cell culture and stimulation

Cell cultures and characterization:. Prior to culture, BAL fluid was filtered, total cell counts were done on a haemocytometer and differential cell counts were performed on cytocentrifuge preparations. Cell pellets were obtained by BAL fluid centrifugation at 400 g for 10 min at 4°C and the cells were resuspended in medium 199 containing 20% FCS with 100 $U \cdot ml^{-1}$ penicillin, 50 $\mu g \cdot ml^{-1}$ streptomycin, and 50 $\mu g \cdot ml^{-1}$ amphotericin B. The number of AM was determined by neutral red staining and viability was assessed by the trypan blue exclusion test. AM (1×10^6) were cultured in Petri dishes (35×10 mm) with 2 ml of medium in a humid atmosphere of 95% air and 5% CO_2 for 2 h at 37°C. The cultures were then washed vigorously three times with warm medium to remove non-adherent cells and to eliminate any remaining polymorphonuclear leucocytes, particularly eosinophils. In some experiments, cells were cultured in Petri dishes on slides and the percentage of macrophages in the adherent cell population was assessed by phagocytosis of latex particles (1.1 μ, 0.1 $mg \cdot ml^{-1}$) after an additional 90 min incubation in 199 medium at 37°C. MGG and toluidine blue staining were used to verify the absence of polymorphonuclear eosinophils or mastocytes in the adherent cells. At the end of each experiment, the adherent cells were scraped gently with a rubber policeman, than counted on a haemocytometer. The results are expressed in terms of this last count.

Cell stimulation. The remaining adherent cells were then incubated in PBS (phosphate buffered saline, pH 7.2) for 5 min at 37°C and stimuli were added. In some experiments, cells were incubated in the presence of calcium ionophore A23187 (2.5 μM) for 30 min. In others, the stimulus was opsonized zymosan (50 $\mu g \cdot ml^{-1}$) added to PBS for an additional 90 min. The opsonization of zymosan was performed according to SELVARAJ *et al.* [31] and resuspended in 0.9% saline; ionophore was diluted in ethanol to a final ethanol concentration of 1% in the culture medium. AM viability was determined by assaying lactate dehydrogenase in an aliquot of the supernatants removed at the end of incubation [32].

Nedocromil sodium incubation. Nedocromil sodium was added to the incubation mixture at the same time as the stimuli, at a final concentration of 10^{-4} M in AM cultures from HS (n=9) and AA (n=10). Dose-response curves from 10^{-7} to 10^{-4} M were plotted only for AM from 3 HS and 5 AA. The response measured was LTB_4 production during stimulation by opsonized zymosan. Cultures incubated under the same conditions but without nedocromil, were used as controls. NDGA, which is considered to be an inhibitor of 5-lipoxygenase activity [33], was also tested at a concentration of 10^{-5} M.

Identification and quantification of AA metabolites

LTB_4 and 5-HETE were assayed in cell free supernatants and in cell suspensions, by HPLC analysis. After specific time intervals, each supernatant was removed and cells were scraped with a rubber policeman into 1 ml of 0.9% saline. An equal volume of filtered HPLC methanol containing prostaglandin B_2 PGB_2 (200 $ng \cdot ml^{-1}$) as an internal standard, was added to cell suspensions and supernatants. After vigorous stirring, the samples were centrifuged and stored at -80°C for further

analysis. Reversed phase HPLC was carried out on a C18 radial pack cartridge (ID 4mm, 10 μ particles) with a Waters model 6000 A pump and U6 K injector. Leukotriene detection was monitored at 280 nm and HETEs at 232 nm, on a Waters model 480 UV absorbance detector. Data were recorded on a Shimadzu CR3-A integrator (Touzart-Matignon). HETEs and LTB_4 were eluted at a flow rate of 1.2 ml·min^{-1} by 2 different isocratic solvent systems, MeOH/H_2O/AcOH (75:25:0.01, v/v/v) and MeOH/H_2O/AcOH (65:35:0.1, v/v/v, adjusted to pH 5.6 with ammonium hydroxide), respectively. Reaction products were identified by comparing their retention times with those of authentic standards. LTB_4 was quantified using PGB_2 as the internal standard, and 5-HETE concentrations were calculated by comparing the area under the peak with those of known amounts of 5-HETE.

The metabolic structures were also identified by their UV spectra obtained on a photoiode array detector (Waters 990), which were compared with those of LTB_4 and 5-HETE standards obtained under the same conditions. Peak homogeneity was evaluated by the symmetry of the isoabsorption recording.

Experimental design and statistical analysis

Data are expressed as mean±SEM. The quantities of AA metabolites are expressed as ng·10^6 AM. The effect of various concentrations of nedocromil used for the dose-response study is expressed as the percentage inhibition of the AM response after drug incubation. Statistical analysis was performed on a VAX 780 computer with the BMPD program. The Mann and Whitney U test [34] was used to determine the significance of variations between AM from HS and AA.

Results

Patients

Morphologic characteristics of patients with asthma are summarized in table 1.

Table 1. – Characteristics of asthmatic patients

Patients no.	Age yrs	Sex	Allergy	FEV_1 % predicted
1	30	M	+	96
2	16	M	+	90
3	37	M	+	100
4	44	F	+	91
5	18	M	+	90
6	19	F	+	98
7	45	M	+	126
8	33	M	+	97
9	41	M	+	75
10	26	F	+	80
11	34	M	+	100
12	41	M	+	110

FEV_1: forced expiratory volume in one second.

Cell populations

The fluid recovery ranged from 40–65%, and was identical in HS and AA. The cell yield per ml of recovered bronchoalveolar fluid was statistically equivalent in the two groups. The percentage of macrophages did not statistically differ between HS and AA. The percentage of eosinophils was significantly higher ($p<0.05$) in BAL fluids, from AA than in those from HS (table 2).

Table 2. – Total and differential cell counts in BAL fluid

	Total cell counts × 1000	Differential cell counts % AM	L	PN	PE
HS (n=11)	240±120	85±17	13±17	1±2	0.1±0.3
AA (n=12)	170±113	83±15	9±8	2±3	4.5±1.3*

*:$p<0.05$; HS: healthy subjects; AA: allergic asthmatics; AM: alveolar macrohages; L: lymphocytes; PN, PE: polymorphonuclear neutrophils and eosinophils, respectively.

In the adherent monolayers, the percentages of AM, as assessed by their morphological characteristics, averaged 98±2%, but only 80±8% of these cells have maintained their ability to phagocytose latex particles. Specific staining showed neither eosinophils nor mastocytes. AM viability in cell cultures from each population at the end of the incubation times was 85±5% as assessed by the trypan blue test, and lactate dehydrogenase (LDH) activity in the medium which was always <3 U·ml^{-1}.

Identification and quantification of AA metabolites

HPLC and UV spectrum analysis. Under our experimental conditions, reversed phase HPLC profiles of cell free supernatants and cells obtained with the eluting solvent systems for LTB_4 or 5-HETE, showed one peak with the same retention time as the authentic sample of LTB_4 (14.50 min) and one with the same retention time as 5-HETE (19.06 min) (fig. 1). The UV spectra of the samples were identical with those of the authentic standards.

AA metabolite determination. When AM were stimulated by ionphore A23187, LTB_4 was released into the supernatant. Its net quantity was greater in AM from AA (AA-AM) than in those from HS (HS-AM) (415±153 *versus* 247±51 ng·10^6 AM, $p<0.05$). No significant difference was observed in the cell content, which was low in both groups (fig. 2a). In contrast, free 5-HETE remained mainly in cells and the level of 5-HETE was higher in AA-AM than in HS-AM (137±28 *versus* 64±16 ng·10^6 AM, $p<0.02$, fig. 2b).

Opsonized zymosan induced a lower synthesis and release of LTB_4 and free 5-HETE than the ionophore. Total LTB_4 was found in equivalent quantities in AM from HS as well as in those from AA, but intracellular LTB_4 levels were slightly higher in AA-AM than in HS-AM (fig. 3a). In contrast, 5-HETE remained mainly in

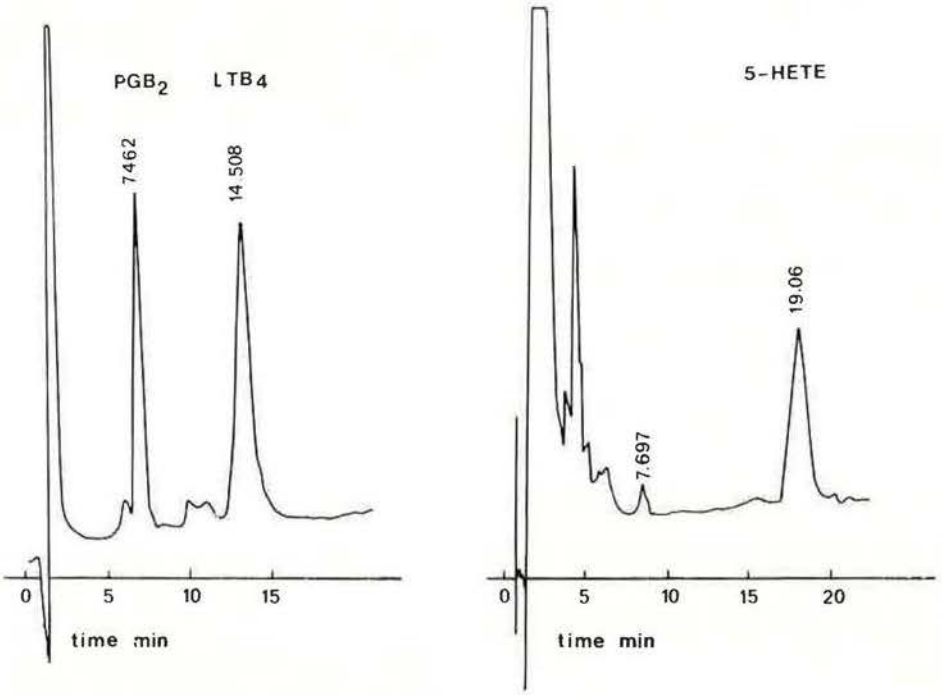

Fig. 1. – Reversed phase high performance liquid chromatography (HPLC) isocratic analysis of arachidonic acid metabolites generated by stimulated alveolar macrophages (AM). Analysis conditions are described in methods. PGB_2: prostaglandin B_2; LTB_4: leukotriene B_4; 5-HETE; 5-hydroxyeicosatetraenoic acid

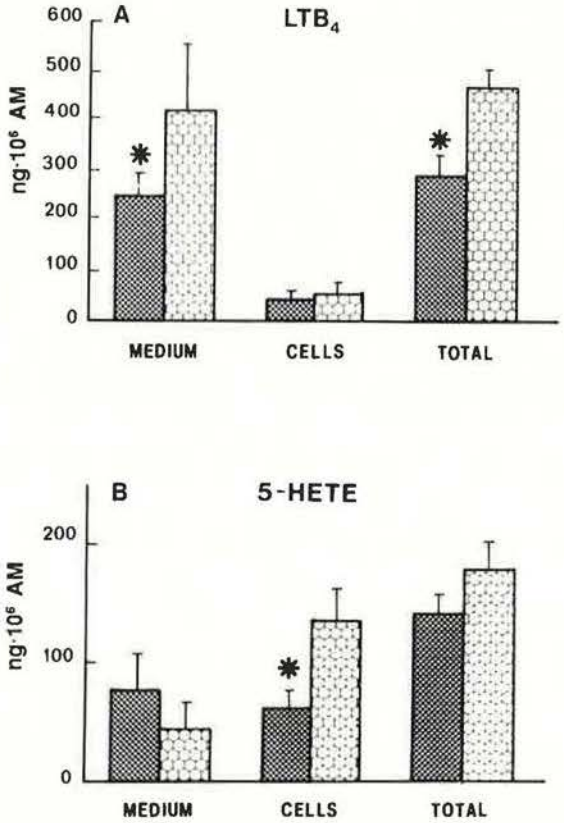

Fig. 2. – Arachidonic acid metabolites generated by AM from healthy subjects (HS ▨) and from asthmatic patients (AA◇) stimulated by ionophore A23187. A: LTB_4 (*:$p<0.05$) B: 5-HETE (*:$p<0.02$). Abbreviation as in figure 1.

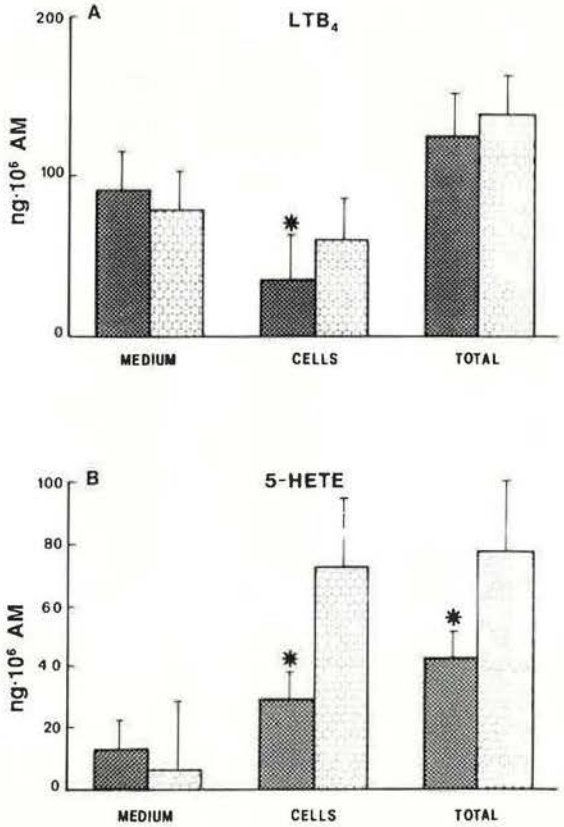

Fig. 3. – Arachidonic acid metabolites generated by AM from healthy subjects (HS ▨) and from asthmatic patients (AA◇) stimulated by opsonized zymosan. A: LTB_4. B: 5-HETE (*:$p<0.05$). Abbreviations as in figuure 1.

the cells and was significantly higher ($p < 0.05$) in AA-AM (fig. 3b).

Nedocromil sodium effect. Nedocromil Na (10^{-4} *M*) did not modify the quantities of LTB_4 and 5-HETE produced after stimulation with ionophore A23187 (data not shown). No difference was observed either in supernatant nor in cells from AA (n=5) nor from HS (n=4).

When AM were stimulated by opsonized zymosan, no change was observed in the quantities of the 2 metabolites in HS-AM (n=9). In contrast, nedocromil induced a significant decrease in LTB_4 and 5-HETE in AA-AM (n=10) (fig. 4a and 4b). Indeed, the levels of LTB_4 synthesized in AM from AA decreased significantly ($p<0.05$) when cells were treated with nedocromil sodium. Before and after treatment the amounts were 61±12 and 45±13 ng·10^6 AM, respectively. In the same way, 5-HETE amounts found in AM were more significantly decreased ($p<0.03$) after nedocromil sodium treatment than these of LTB_4. 5-HETE averaged 72±15 and 53±17 mg·10^6 AM before and after nedocromil sodium treatment, respectively. Thus, total LTB_4 and 5-HETE release was also decreased.

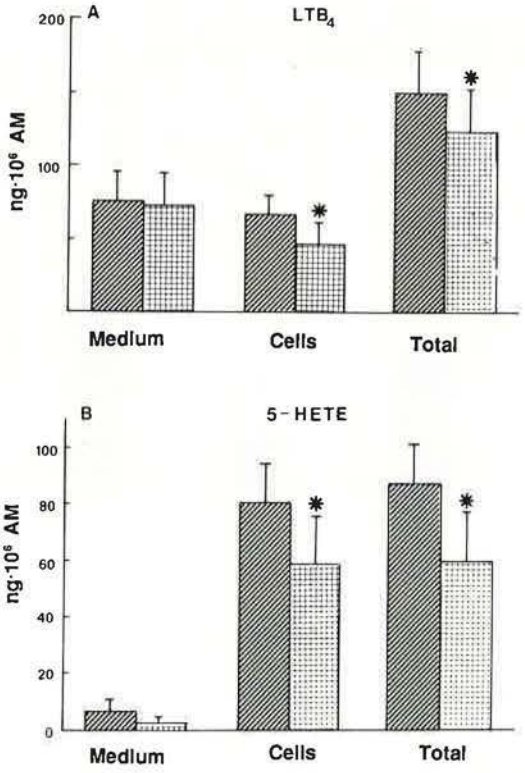

Fig. 4. – Effect of nedocromil sodium (10^{-4} M) on arachidonic acid metabolites generated by AM from asthmatic patients (AA) stimulated by opsonized zymosan (▨: control, ▦: nedocromil). A: LTB_4 (*:$p<0.05$). B: 5-HETE (*p:<0.03). Abbreviations as in figure 1.

Under the same conditions, NDGA significantly decreased (80% of metabolite generation in control cultures, $p<0.001$) the quantities of LTB_4 and 5-HETE released and synthesized by AM from the two populations stimulated with the ionophore or opsonized zymosan.

The dose-response study showed that nedocromil sodium had no effect on AM from HS. On the other hand, a dose-dependent effect was observed in AM from 4 out of 5 AA tested (fig. 5); thus the maximal inhibition of LTB_4 release was observed for a concentration of 10^{-4} *M*.

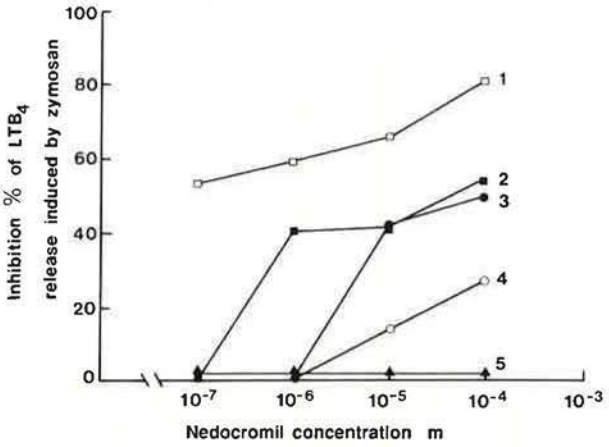

Fig. 5. – Dose-response curves obtained with nedocromil sodium in AM from 4 out of 5 asthmatic patients. AM were stimulated by opsonized zymosan. The results are expressed as percent of inhibitory effect promoted by nedocromil sodium. Abbreviations as in figure 1.

Discussion

We first compared the capacity of stimulated AM from healthy subjects and asthmatic patients to generate LTB_4 and 5-HETE from endogenous arachidonic acid, and then checked the *in vitro* effect of nedocromil sodium. In a previous report, we have shown that AM from the two populations can be activated by calcium ionophore or opsonized zymosan [9]. These findings are in agreement with those of CHANG *et al.* [35], MACDERMOT *et al.* [36], and LAVIOLETTE *et al.* [37] who have shown that activated AM are able to generate inflammatory mediators.

In the present study, the patients were in stable condition (as assessed by forced expiratory volume in one second (FEV_1) and we found that calcium-ionophore-stimulated AM from asthmatic patients released more LTB_4 than those from healthy volunteers. No difference was observed between the quantities of LTB_4 contained in the cells. The same data were obtained when AM were stimulated with opsonized zymosan, but the quantities were smaller than in ionophore-stimulated-cultures. The release of LTB_4 was slightly higher in AM from AA than those in HS. These data are in agreement with the concept of releasability, which has been described in peripheral blood cells such as basophils and neutrophils [38, 39], and in BAL fluid, whose mast cells and eosinophils were activated to release mediators [40, 41]. FLINT *et al.* [42] found a close relationship between bronchoalveolar mast cell releasability and bronchial hyperreactivity (BHR) in asthma. Oxygen species releasability in AM has been correlated with the severity of asthma. This mediator releasability in bronchial asthma seems to be important since this process may be correlated with bronchial hyperreactivity, and with the severity of the

disease, as determined by symptom score [24]. Since the highest releasability of LTB_4 was found in AM from AA, these cells may participate in the initiation and amplification of the inflammatory process characterizing asthma, considering that this leukotriene has been reported to exert potent biological functions in asthma. Specifically, LTB_4 is a potent chemotactic factor for neutrophils and eosinophils [43]; it is able to enhance the proliferation of supressor-cytotoxic T-cells, and inhibit the proliferation of helper-inducer T-lymphocytes [44–46]. It also enhances lymphocytotoxicity and interferon production [47]. Some of these abnormalities have been described in asthmatic patients [48, 49].

In contrast, free 5-HETE was higher in the cells stimulated by the ionophore or opsonized zymosan compared to their supernatant, and was higher in cells from AA than in those from HS. This observation is in agreement with results of PAWLOWSKI *et al.* [50] who found that mouse peritoneal macrophages incorporated exogenous mono-HETEs. This reaction was specific, since the macrophages failed to take up other oxygenated derivatives of arachidonic acid. On the other hand, these authors showed that the uptake of 5-HETE was increased only when cells were activated *in vivo*, and it was not metabolized [51]. This uptake suggests a possible physiological role for 5-HETE in regulating macrophage function. Such a role could be identical to that suggested for eosinophils by GOETLZ *et al.* [52], who showed that the preservation of a normal cellular concentration of endogenous HETEs is required for cells to achieve optimal random migration and to respond to other chemotactic factors. Specifically, when lipoxygenase inhibitors depleted the eosinophil HETE content, random migration and chemotaxis were significantly inhibited; addition of purified native HETEs restored migration to normal levels. Since inflammatory macrophages are more responsive to chemotactic factors than resident cells [53], the increase in intracellular free 5-HETE in AM from asthmatics observed under our experimental conditions, may be correlated with the migratory ability of these activated macrophages.

Nedocromil sodium decreased the generation of LTB_4 and 5-HETE only in AM from asthmatic patients. No effect was observed in AM from healthy volunteers. In AM from the asthmatic patients, the action of nedocromil sodium was dose-dependent. It significantly decreased intracellular free 5-HETE. Its clinical efficacy [54] may be due to its action on cells of the bronchoalveolar lumen. These results are in agreement with those obtained with other activated cells: inhibition of the complement-dependent release of eosinophil granule proteins [55], human granulocyte activation [54], IgE-dependent rat macrophage and platelet activation [56], and anti-IgE-induced-histamine release from mast cells [57–59]. Thus, this drug may suppress cell activation and mediator release, and thereby prevent local inflammatory reaction and secondary tissue damage.

In a further work, the role of intracellular free 5-HETE will be investigated by studying the chemotactic ability of AM from AA and HS, and its response to the action of nedocromil sodium.

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L'augmentation des métabolites arachidonique LTB_4 et 5-HETE des macrophages alveolaires des asthmatiques: effet in vitro de nedocromil sodium. M. Damon, C. Chavis, J.P. Daures, A. Crastes de Paulet, F.B. Michel, Ph. Godard.

RÉSUMÉ: Les macrophages alvéolaires, principales cellules phagocytaires de la lumière bronchique, jouent un rôle important dans les réactions de défense pulmonaire. Dans l'asthme, ces cellules participent à l'amplification du processus inflammatoire. Les macrophages activés libèrent du leucotriène B_4 et du 5-HETE, deux médiateurs pharmacologiquement actifs. Ces deux métabolites de l'acide arachidonique sont identifiés et dosés par HPLC dans les cellules et dans les surnageants correspondants. Les macrophages des asthmatiques, après une stimulation par le ionophore ou le zymosan opsonisé libèrent une quantité plus importante de LTB_4 que les cellules des sujets normaux. L'augmentation de cette capacité à libérer du LTB_4 reflète l'état d'activation des cellules in vivo. Les taux intracellulaires de 5-HETE sont plus élevés dans le cas des sujets asthmatiques. Cette élévation de taux pourraît être correlée avec une plus grande capacité des cellules à se déplacer. L'efficacité du Nedocromil pourraît être fonction de son action sur le taux intracellulaire du 5-HETE et sur la capacité des macrophages à libérer du LTB_4.

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