

## Generation of oxygen free radicals from blood eosinophils from asthma patients after stimulation with PAF or phorbol ester

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**ABSTRACT:** Eosinophils (EOS) may play an important role in the pathophysiology of bronchial asthma because they can release oxygen free radicals and several basic proteins which are cytotoxic to bronchial epithelium. We have studied the response of EOS isolated from the blood of atopic subjects with symptoms of asthma (AS, n=7) or rhinitis (AR, n=4) or without symptoms (AA, n=5) and of subjects with the hypereosinophilic syndrome (HES, n=5). EOS were separated using metrizamide density gradients and activated *in vitro* with platelet-activating factor (PAF, 100 nM) or phorbol 12-myristate-13-acetate (PMA, 100 nM). Oxygen free radical generation was assessed by a lucigenin-enhanced chemiluminescence (CL) assay. EOS purity was  $83 \pm 1.7\%$  (mean  $\pm$  SEM) with greater than 95% viability. Background CL responses of EOS from HES were significantly higher than those from AA ( $p < 0.01$ ) and AR ( $p < 0.05$ ). Normodense EOS from AS (PAF-induced CL =  $90 \pm 27$  mV) were more responsive to PAF than were those from AR ( $17 \pm 13$  mV,  $p < 0.01$ ) and from AA ( $23 \pm 14$  mV,  $p < 0.01$ ). Similar results were obtained with PMA. Hypodense EOS from HES subjects were as responsive as normodense EOS from AS to PMA and PAF. Thus, EOS from AS have an enhanced potential for activation, particularly by PAF; this may represent an important mechanism for the perpetuation of the inflammatory response in asthma, since EOS can also release PAF.

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Eosinophil leukocytes are conspicuous within the airway wall of patients suffering from asthma [1]. In addition, increased numbers of eosinophils can be recovered from bronchoalveolar lavage fluid of asthmatic subjects, with a further increase in recovery of these cells after allergen provocation [2]. Recent work on the pathophysiology of the eosinophil has cast light upon the potential role of this inflammatory cell in asthma [3]. Eosinophils have the capacity to release cationic proteins such as major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) as well as oxygen free radicals, which have been shown to be cytotoxic to airway cells such as the bronchial epithelium [4, 5]. In addition, eosinophils may generate newly-formed mediators derived from the metabolism of membrane phosphoglycerides, for example leukotriene C<sub>4</sub> (LTC<sub>4</sub>) and platelet-activating factor (PAF) [6, 7].

The capability of eosinophils to release PAF, particularly when stimulated by immunoglobulin-dependent mechanisms [8], is of particular relevance to the pathophysiology of asthma because PAF is not

only a potent bronchoconstrictor agent but is also capable of inducing bronchial hyperresponsiveness in man [9, 10], a characteristic feature of asthma. We have shown recently that PAF induces release of the granule enzyme eosinophil peroxidase (EPO) from human eosinophils [11]. PAF is also one of the most potent chemotactic agents for eosinophils *in vitro* [12] and can activate eosinophils to release oxygen free radicals and LTC<sub>4</sub> [13]. These interactions between eosinophils and PAF may form the basis of self-amplifying mechanisms for the inflammatory changes observed in the airways of asthmatic patients.

In the present study, we have examined whether circulating eosinophils obtained from symptomatic atopic asthmatic subjects display increased reactivity to PAF *in vitro*. We compared these responses with those of eosinophils obtained from atopic subjects with and without symptoms of rhinitis and from patients with the hypereosinophilic syndrome. In addition, we studied the responses of these eosinophils to the protein kinase C activator, phorbol 12-myristate-13-acetate (PMA).

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Table 1. — Characteristics of patients

Patient no.	Age yrs	Sex	Diagnosis	FEV <sub>1</sub> * % predicted	Leucocyte count ×10 <sup>9</sup> .l <sup>-1</sup>	Eosinophil count ×10 <sup>6</sup> .l <sup>-1</sup> (%)	Medication
1	25	M	AS	73.7	7.1	915 (13)	A, B
2	55	F	AS	42	5.6	336 (6)	A
3	71	F	AS	70	11.5	2070 (18)	A
4	30	F	AS	55	12.7	1397 (11)	A, B
5	40	M	AS	61	11.1	1776 (16)	A, B, T
6	25	M	AS	37	8.5	765 (9)	A
7	20	F	AS	37.1	5.8	580 (10)	A, B, T
8	56	M	HES	ND	13.2	10032 (76)	P
9	44	M	HES	ND	15.0	9000 (60)	P
10	63	F	HES	ND	7.5	5475 (73)	P
11	55	F	HES	ND	5.5	1925 (35)	P
12	30	M	HES	ND	9.5	4158 (44)	-
13	23	F	AR	96.1	5.1	204 (4)	-
14	34	F	AR	90	5.4	486 (9)	-
15	22	F	AR	138	5.7	570 (10)	-
16	35	M	AR	100	6.7	536 (8)	-
17	43	M	AA	94	5.3	318 (6)	-
18	28	M	AA	97	6.6	198 (3)	-
19	37	M	AA	120	4.6	460 (11)	-
20	27	M	AA	114	5.7	399 (7)	-
21	27	M	AA	83	6.3	567 (9)	-

AS: atopic asthmatic; HES: hypereosinophilic syndrome; AR: atopic rhinitis; AA: asymptomatic atopics; \*: according to Quanjer, *Clin Respir Physiol*, 1983, 19, (Suppl. 5); ND: not determined; A: albuterol aerosol; B: beclomethasone propionate aerosol; T: theophylline; P: prednisolone.

## Methods

### Subjects

Four different groups of subjects were studied: symptomatic atopic asthmatics (AS), symptomatic rhinitic atopics (AR), asymptomatic atopics (AA) and hypereosinophilic syndrome (HES). Atopy was defined as giving a positive wheal and flare response to skin prick testing with several common allergens including extracts from grass pollen, cat and dog hair and house dust mite. The characteristics of the subjects, including forced expiratory volume in one second (FEV<sub>1</sub>) and total leucocyte and eosinophil counts were measured on the day of study (table 1). Asthmatic subjects were all symptomatic with increasing wheeze - not controlled by their current medication - at the time of study, and had had asthma diagnosed according to the criteria of the American Thoracic Society for at least 10 yrs [14]. None of the subjects had been on oral corticosteroids for at least one month, but all were taking daily inhaled beta-adrenergic agents, four taking inhaled beclomethasone dipropionate and two taking oral theophylline preparations. Subjects currently suffering from atopic rhinitis were studied during the hayfever season, with symptoms of blocked and runny noses and sneezing for at least 1 mth prior to study. Asymptomatic atopic subjects denied any symptoms of rhinitis, eczema or asthma, although 3 subjects had had seasonal symptoms of rhinitis in the past. Patients with the hypereosinophilic syndrome all had long-standing peripheral eosinophilia greater than 4,000·mm<sup>-3</sup> for at least 1 year with no obvious secondary cause found

[15]; four of the five patients were taking prednisolone daily (range 10–20 mg per day).

### Eosinophil separation

Venous blood was obtained from a forearm vein through a 19G cannula into heparinized syringes (100 units heparin per 50 ml blood) and added to 1/5 volume of Dextran 110 (6% in 0.9% NaCl; Fisons plc, Loughborough, UK) in sterile polystyrene tubes. Sedimentation of red blood cells was allowed for 45 min at 37°C and the leucocyte-rich plasma was removed. The mixed leucocytes were washed twice and resuspended in Hank's balanced salt solution (HBSS; Flow Laboratories, Irvine, UK), the final suspension being supplemented with 10% fetal calf serum (Sigma Chemical Co, Poole, UK).

Discontinuous density gradients were prepared from metrizamide (Nycomed AS, Oslo, Norway). A stock solution of 30% (w/v) metrizamide in Tyrode's solution containing 10 g·l<sup>-1</sup> gelatine and 30 mg·l<sup>-1</sup> deoxyribonuclease I (Sigma) (TGD) was diluted with TGD to give solutions of 25%, 24%, 23%, 22%, 20% and 18%. Six-step gradients were prepared by careful layering of 2 ml volumes of decreasing densities and of metrizamide solutions in 15 ml conical centrifuge tubes (Becton-Dickinson Labware, New Jersey, USA) as described previously [16, 17]. Leucocyte suspensions were layered on top of the metrizamide gradients and the tubes were centrifuged at room temperature for 45 min at 1,300 × g. Cells were collected at each interface and washed twice with HBSS; residual erythrocytes could



be removed at this stage by hypotonic lysis (suspension in ice-cold distilled water for 30 s). Each cell pellet was resuspended in HEPES buffer - containing HBSS supplemented with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Sigma) and 3.5 g.l<sup>-1</sup> bovine serum albumin (Sigma) - and counted in a Neubauer haemocytometer after staining with Kimura stain [18]. Eosinophil-rich fractions were adjusted to a cell concentration of  $3.33 \times 10^6$  cells.ml<sup>-1</sup> and stored at 37°C for at least 15 min prior to use. The percentage of eosinophils was determined by cyto-centrifugation and staining of prepared slides with May-Grünwald-Giemsa stain. Viability was assessed by exclusion of trypan blue. Eosinophils obtained from the low density layers (between 1.115 and 1.125 g.ml<sup>-1</sup>, 22–23% metrizamide) were classified as hypodense cells and those from the higher density layers (1.125–1.130 g.ml<sup>-1</sup>, 23–25% metrizamide) as normodense. The purity of eosinophil preparations ranged between 70–95% (AA 85±3.3%, AR 76±3.1%, AS 82±2.8% and HES 88±3.1%) the sole contaminating cell type being neutrophils. Viability of the preparations was greater than 95%.

#### Chemiluminescence (CL) assay

Bis-N-methylacridinium nitrate (Lucigenin; Sigma) and PMA (Sigma) were dissolved to 10 mM in dimethylsulphoxide (DMSO; Sigma) and stored at -20°C. Superoxide dismutase (SOD; Sigma) and nordihydroguaiaretic acid (NDGA, Sigma) were dissolved to 3 mg.ml<sup>-1</sup> and 1 mM, respectively, in DMSO and stored at -20°C. PAF (hexadecyl form; Bachem Feinchemikalien AG, Bubendorf, Switzerland) was dissolved to 10 mM in 100% ethanol and stored at -70°C. WEB 2086 (kindly donated by Boehringer Ingelheim, Germany) was dissolved initially in 1 M HCl and diluted in HEPES buffer in concentration ranges of 10<sup>-9</sup> to 10<sup>-4</sup> M [19]. All compounds were diluted in HEPES buffer.

Aliquots of eosinophil suspension (300 µl) were added to 5 ml polystyrene cuvettes (Clinicon; LKB-Pharmacia, Milton Keynes, UK) containing 500 µl lucigenin (final concentration 50 µM), 100 µl HEPES buffer and 100 µl PAF or PMA (final concentrations 100 nM) or HEPES buffer. In some experiments, 100 µl HEPES buffer was replaced with SOD (30 µg.ml<sup>-1</sup>) or NDGA (100 µM). The cuvettes were introduced into the carousel of a luminescence photometer (Luminometer 1251; LKB-Wallace OY, Turku, Finland), thermostat set at 37°C, and luminescence measurements made at regular intervals for 30 min. In a previous study we have shown that PAF (100 nM) and PMA (100 nM) did not cause significant release of lactate dehydrogenase, indicating lack of cytotoxic effects [20]. To determine the effect of the specific PAF antagonist WEB 2086, cells were preincubated with WEB 2086 or HEPES buffer for 2 min prior to stimulation with PAF (100 nM) or PMA (100 nM).

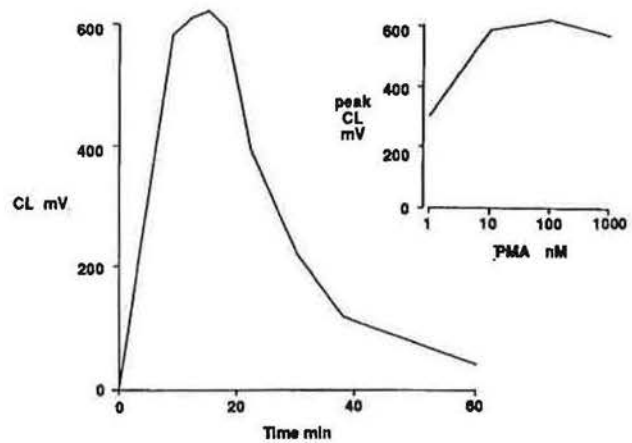


Fig. 1. - Time-course of chemiluminescence (CL) in eosinophils ( $10^6$  in 1 ml) obtained from an asthmatic subject and stimulated with PMA (100 nM). Inset: concentration-response relationship of the CL response showing a maximal effect at 100 nM.

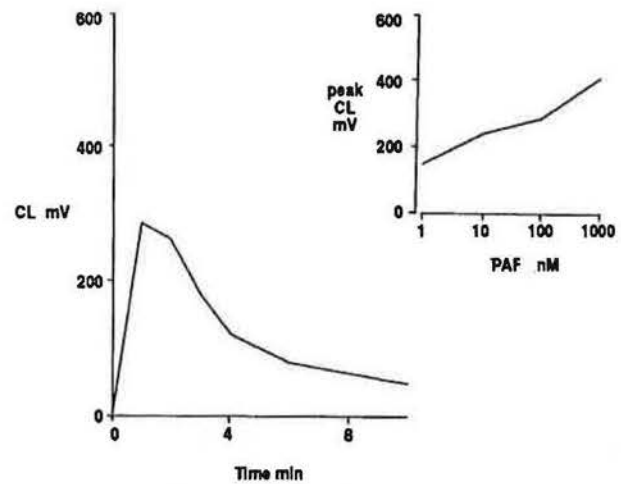


Fig. 2. - Time-course of chemiluminescence (CL) in eosinophils ( $10^6$  in 1 ml) obtained from an asthmatic subject and stimulated with PAF (100 nM). Inset: concentration-response relationship of the CL response.

#### Statistical analysis

Data are reported as mean±SEM for each group. Pairwise comparisons between groups were made by Student's t-test using a within-group pooled estimate of variance [21]. To determine whether there was any linear relationship between the CL responses of eosinophils and peripheral eosinophil count or lung function, Pearson correlation coefficients (*r*) were calculated. Statistical significance was defined as *p*<0.05.

#### Results

We studied normodense eosinophils from asthmatic, rhinitic and asymptomatic subjects - the hypodense fractions being insufficiently pure in eosinophils - while hypodense fractions from patients with the



hypereosinophilic syndrome were used, only these fractions being sufficiently rich in eosinophils.

The mean spontaneous CL of eosinophils from the HES subjects ( $101 \pm 56$  mV) was higher than that of AA ( $3 \pm 1.8$  mV,  $p < 0.01$ ) or AR ( $3.4 \pm 1.7$  mV,  $p < 0.05$ ) subjects. Baseline CL of AS eosinophils ( $50 \pm 18$  mV) was not significantly different from that of AA or AR.

The CL responses to PAF and PMA were time- and dose-dependent (figs 1 and 2). The time courses of the responses induced by the two stimuli were different, the PMA-induced CL being slower to peak and more prolonged than that induced by PAF. In all subjects, PMA 100 nM produced a greater luminescence than PAF at the same concentration; the PMA and PAF responses in individual subjects bearing a direct relationship to each other ( $r = 0.70$ ,  $n = 21$ ,  $p < 0.01$ ). The CL induced by PMA and PAF was inhibited partially by SOD ( $30 \mu\text{g}\cdot\text{ml}^{-1}$ ; inhibition  $37 \pm 8\%$ ,  $n = 3$ ) and completely by the antioxidant NDGA ( $10 \pm 0 \mu\text{M}$ ; inhibition 99%,  $n = 2$ ). PAF-induced CL was inhibited by the PAF antagonist, WEB 2086, in a dose-dependent manner with a median inhibitory concentration ( $IC_{50}$ ) of  $3.54 \pm 1.81 \mu\text{M}$  ( $n = 3$ ) against 100 nM PAF. However PMA-stimulated responses were not inhibited by WEB 2086 up to  $10^{-4}$  M.

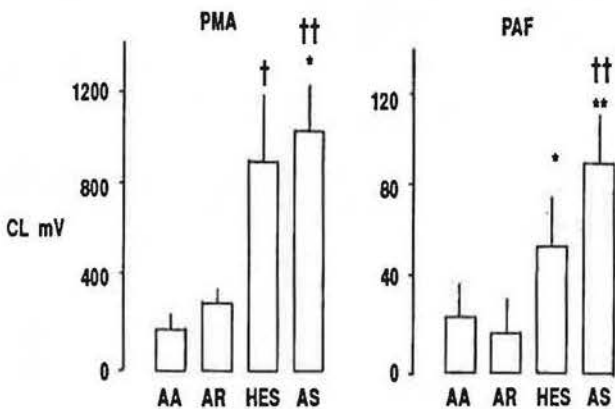


Fig. 3. - Chemiluminescence (CL) response (mean  $\pm$  SEM) of eosinophils obtained from asymptomatic atopic subjects (AA;  $n = 5$ ), allergic rhinitis subjects (AR;  $n = 4$ ), patients with the hypereosinophilic syndrome (HES;  $n = 5$ ), and symptomatic asthmatic patients (AS;  $n = 7$ ): left, stimulated with PMA (100 nM); right, stimulated with PAF (100 nM). †:  $p < 0.05$ ; ††:  $p < 0.01$  compared to AA; \*:  $p < 0.05$ ; \*\*:  $p < 0.01$  compared to AR.

Pairwise comparisons showed that PMA-induced CL of eosinophils obtained from asthmatic subjects was significantly higher than that of cells obtained from rhinitic atopics ( $p < 0.05$ ) and asymptomatic atopics ( $p < 0.01$ ) (fig. 3). Thus, mean CL in asthmatic subjects was  $1032 \pm 285$  mV compared to  $286 \pm 59$  mV in rhinitic and  $177 \pm 70$  mV in asymptomatic subjects. Hypodense eosinophils from HES patients were more responsive to PMA than the normodense eosinophils from asymptomatic atopics ( $p < 0.05$ ) but were not significantly different from rhinitic or asthmatic patients' cells.

Similar results were obtained with PAF-induced CL (fig. 3). Eosinophils from asthmatics were more

responsive (CL  $90 \pm 27$  mV) than those obtained from rhinitic ( $17 \pm 13$  mV,  $p < 0.01$ ) and asymptomatic atopics ( $23 \pm 14$  mV,  $p < 0.01$ ). Hypodense eosinophils from HES patients showed significantly higher responses ( $54 \pm 23$  mV) than normodense cells from atopic rhinitic subjects ( $p < 0.05$ ).

In order to exclude the possibility that the contaminating neutrophils might have contributed to the enhanced CL observed in the asthmatic subjects, we compared lucigenin-enhanced CL of neutrophils ( $>95\%$  purity and viability) isolated from 3 of the asthmatic subjects with those from 3 of the asymptomatic atopics. Peak CL responses to PMA (100 nM) were  $475 \pm 68$  mV for AA, compared with  $492 \pm 48$  mV for AS (NS), and responses to PAF (100 nM) were  $14 \pm 5.5$  mV for AA, compared to  $11 \pm 2.3$  mV for AS (NS).

## Discussion

We have shown that eosinophils isolated from the blood of symptomatic asthmatic subjects are approximately three times as responsive in terms of chemiluminescence response to the exogenous stimuli PAF and PMA, when compared with eosinophils obtained from allergic symptomatic rhinitic or allergic asymptomatic subjects. The responsiveness of the normodense eosinophils from symptomatic asthmatics was similar to that of the hypodense eosinophils from patients with the hypereosinophilic syndrome. Although neutrophils were consistently present in the eosinophil preparations, they were unlikely to contribute to the hyperresponsiveness of the cells obtained from symptomatic asthmatic subjects because pure neutrophil preparations from the same subjects did not show a higher response than those obtained from asymptomatic allergic subjects. In addition we found higher levels of background activity in eosinophils purified from patients with asthma and the hypereosinophilic syndrome.

Enhanced eosinophil metabolic activity has been reported previously in patients with the hypereosinophilic syndrome [22, 23]. In these studies, hypodense eosinophils, which have been shown to possess an increase in membrane receptor expression [24, 25], to release more granule proteins [24] and to have a greater helminthotoxic capacity [26] than normodense eosinophils, were studied. In a more recent study, FUKUDA *et al.* [27] found increased proportions of hypodense eosinophils in patients with asthma. It is unlikely that we were studying hypodense eosinophils in our asthmatic subjects since we isolated cells from the lowest step gradient interface, which we and others have defined as containing normodense eosinophils [17]. A similar procedure was carried out in the allergic symptomatic and asymptomatic subjects. In addition, SHULT *et al.* [28] have not found a heterogeneous density pattern of eosinophils in the blood of patients with allergic rhinitis.

The allergic subjects we studied had increased numbers of circulating eosinophils and, in particular, the asthmatic subjects had a significantly higher number



than the allergic symptomatic and asymptomatic subjects. This is not surprising because we deliberately studied asthmatic subjects during an exacerbation of their symptoms which can be associated with a peripheral eosinophilia [29]. We found no correlation between the chemiluminescence response to either PAF or PMA and the eosinophils count, suggesting that the underlying cause for eosinophilia was not directly related to the enhanced oxidative metabolism observed in the asthmatic subjects. SHULT *et al.* [28] found an increased responsiveness of the chemiluminescence response of eosinophils isolated from the blood of symptomatic allergic rhinitis subjects to opsonized zymosan and to PMA when compared to those of normal subjects; the increased response to zymosan persisted even when the subjects were asymptomatic. Taken together, the present study and that of SHULT *et al.* [28] suggest that eosinophils from allergic asymptomatic asthmatics are even more responsive than those from allergic symptomatic rhinitis subjects when compared to normal subjects. The enhanced metabolic response of eosinophils from asthmatics is not only confined to this cell type because CLUZEL *et al.* [30] have recently reported that the luminol-enhanced chemiluminescence response of alveolar macrophages from asthmatic subjects is increased and is related to the severity of asthma. However, neutrophils do not share this metabolic enhancement in either asthma or rhinitis.

The mechanism of enhanced response of the eosinophil in asthma remains to be determined. The oxidative metabolic activation of the eosinophil by PAF is likely to be receptor-mediated because of its inhibition by the PAF antagonist WEB 2086 [20]. PAF receptor activation may lead to an increase in inositol phosphate turnover [31]. Thus, an increase in the number of PAF receptors or in their affinity or in the efficiency of receptor coupling mechanisms may underlie the increased metabolic activity of eosinophils from asthma. The concomitant increase in response to PMA of the eosinophils suggests that an intracellular mechanism involving protein kinase C activation may also be involved. Several cytokines such as colony-stimulating factor [32], eosinophil differentiating factor [33] and tumour necrosis factor [34] have been shown to activate and enhance eosinophil responses *in vitro*. It is possible that release of these cytokines in asthma may prime eosinophils to increase their oxidative metabolism. These cytokines are also able to convert normodense eosinophils to hypodense eosinophils possessing the augmented biochemical properties of the hypodense eosinophils from patients with the hypereosinophilic syndrome [35]. In addition, hypodense eosinophils have been described in asthmatic subjects. However, these cytokines can stimulate both eosinophils and neutrophils [32] and may not, therefore, be the only mechanism implicated in the more specific enhancement of eosinophil function in symptomatic asthma.

The enhanced response of eosinophils obtained from symptomatic asthmatic subjects further supports a role for this cell in asthma. The involvement of PAF in the

pathophysiology of asthma is still under investigation [36]. We have recently demonstrated that a PAF antagonist, BN 52063, significantly inhibited the late phase induration after cutaneous antigen challenge in atopic subjects [37]. This suggests a role for endogenously-released PAF in causing cellular infiltration, presumably of eosinophils, provoked by an immunoglobulin E-mediated response. The enhanced response of the asthmatic eosinophil to endogenously-released PAF may constitute an important amplification pathway for the induction of inflammation characteristic of the asthmatic airway.

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- Production de radicaux libres d'oxygène par les éosinophiles du sang, chez des patients asthmatiques après stimulation par le PAF ou par l'ester de phorbol.* P. Chanez, G. Dent, T. Yukawa, P.J. Barnes, K.F. Chung.
- RÉSUMÉ: Les éosinophiles (EOS) peuvent jouer un rôle important dans la physiopathologie de l'asthme bronchique, parce qu'ils peuvent libérer des radicaux libres d'oxygène et différentes protéines basiques qui sont cytotoxiques pour l'épithélium bronchique. Nous avons étudié la réponse d'éosinophiles isolés du sang de sujets atopiques avec des symptômes d'asthme (AS, n=7) ou de rhinite (AR, n=4), ou de sujets sans symptôme (AA, n=5), ainsi que de sujets porteurs d'un syndrome d'hyperéosinophilie (HES, n=5). EOS ont été isolés par des gradients de densité à la metrizamide, et activés *in vitro* par le facteur d'activation des plaquettes (PAF, 100 nM) ou le phorbol 12-myristate-13-acétate (PMA, 100 nM). La production de radicaux libres d'oxygène a été mesurée par un essai de chemoluminescence accentué par la lucigenine (CL). La pureté des éosinophiles atteignait 83±1.7%, avec une viabilité supérieure à 95%. Les réponses de base de CL des éosinophiles provenant de HES sont significativement plus marquées que celles provenant de AA (p<0.01) ou de AR (p<0.05). Des éosinophiles normodenses provenant de AS (chemoluminescence induite par le PAF = 90±27 mV) ont répondu davantage au PAF que ceux provenant des AR (17±13 mV, p<0.01) ou provenant de AA (23±14 mV, p<0.01). Des résultats similaires ont été obtenus au moyen de PMA. Les éosinophiles hypodenses provenant de sujets HES s'avèrent aussi réactifs que les éosinophiles normodenses provenant de AS, à l'égard de PMA et de PAF. Donc, les éosinophiles provenant de AS ont une potentialité accentuée d'activation, particulièrement sous l'effet du PAF; ceci pourrait représenter un important mécanisme pour la perpétuation de la réponse inflammatoire dans l'asthme, puisque les éosinophiles peuvent également produire du PAF. *Eur Respir J.*, 1990, 3, 1002–1007.