

## Products of neutrophils and eosinophils increase the responsiveness of human isolated bronchial tissue

A.R. Hallahan, C.L. Armour, J.L. Black

*Products of neutrophils and eosinophils increase the responsiveness of human isolated bronchial tissue. A.R. Hallahan, C.L. Armour, J.L. Black.*

**ABSTRACT:** This study examines the possibility that products of neutrophils and eosinophils could increase the responsiveness of human isolated bronchial tissue. Neutrophils and eosinophils were isolated from the peripheral blood of healthy volunteers. The cells were incubated with  $1 \mu\text{M}$  calcium ionophore A23187 for 10-15 min then centrifuged, the supernatant collected and stored at  $-70^\circ\text{C}$ . Human bronchial rings (2-3 mm diameter, 3-4 mm long) were prepared from specimens resected at thoracotomy. The tissues were suspended in organ baths under a 1 g load and changes in tension measured isometrically. Stable contractions to bolus doses of histamine ( $0.1-10 \mu\text{M}$ ) or to electrical field stimulation (40-100 V, 4-16 Hz, 1 ms for 20 s) were established. Supernatant from 106 neutrophils or 105 eosinophils was then added and tissue responsiveness reassessed. Neutrophil supernatant increased tissue responsiveness to histamine and electrical field stimulation by  $54 \pm 17\%$  ( $n=5$ ,  $p<0.05$ ) and  $18 \pm 7\%$  ( $n=6$ ,  $p<0.05$ ), respectively. Eosinophil supernatant increased the histamine response by  $60 \pm 23\%$  ( $n=8$ ,  $p<0.05$ ) while tissue responsiveness to electrical field stimulation was unchanged ( $n=3$ ). Thus, as neutrophils and eosinophils can change the responsiveness of human bronchus *in vitro* it is possible that they do this *in vivo* and may not simply be temporally related to the development of bronchial hyperresponsiveness.

*Eur Respir J.*, 1990, 3, 554-558.

Depts of Pharmacology and Pharmacy, University of Sydney, New South Wales, Australia.

Correspondence: C.L. Armour, Department of Pharmacy, University of Sydney, N.S.W. 2006 Australia.

Keywords: Asthma; bronchi; eosinophils; histamine; neutrophils.

Supported by the National Health and Medical Research Council of Australia and the Asthma Foundation of N.S.W.

Bronchial hyperresponsiveness (BHR) is a characteristic feature of asthma but the mechanisms of BHR are not known [1]. BHR may be due to an abnormality in the airway smooth muscle. *In vivo* and *in vitro* airway responsiveness correlate in a dog model of hyperresponsiveness [2] but not in humans [3-5]. However, in these human studies removal of the tissue from its inflamed environment *in vivo* and study some time later may have resulted in the loss of short lived inflammatory mediators critical for the development of hyperresponsiveness *in vitro*.

Airway inflammation is strongly implicated as the cause of BHR but the roles of the different types of inflammatory cells remain controversial. The actions of neutrophils and eosinophils on airways have been of particular interest. Neutrophils are associated with some acute models of BHR [6] but raised levels are not seen in stable asthma [7]. This suggests that airway inflammation may only transiently increase airway responsiveness. Eosinophil levels are raised in bronchoalveolar lavage fluid from subjects with asthma and the degree to which this occurs correlates with disease severity [8]. Whether they cause the disease or are simply associated with it is unknown.

Inflammatory cell products have been shown to increase airway responsiveness in some animal models [9, 10] but not others [11]. However, the effects of inflammation on human isolated airways have not been previously examined. Thus, this study investigated the possibility that neutrophil and eosinophil products could increase the responsiveness of human isolated bronchial tissue to histamine and electrical field stimulation.

### Methods

#### *Neutrophil purification and activation*

Neutrophils were purified from the venous blood of healthy volunteers to  $96.0 \pm 0.4\%$  purities and  $>98\%$  viabilities using a modification of the method developed by FERRANTE and THONG [12]. Blood was collected into lithium-heparin coated tubes, layered onto Mono-Poly Resolving Medium (Flow Laboratories, Sydney, Australia) in plastic centrifuge tubes and centrifuged at

300 g for 75 min. The neutrophils separated into a distinct cell band. They were collected, washed once in Hank's balanced salt solution without calcium or magnesium (HBSS) (Commonwealth Serum Laboratories, Parkville, Australia) and resuspended in Krebs-Henseleit solution (composition, mmol·l<sup>-1</sup>: Na<sup>+</sup>, 143.4; K<sup>+</sup>, 5.9; Ca<sup>2+</sup>, 2.5; Mg<sup>2+</sup>, 1.2; Cl<sup>-</sup>, 128.1; SO<sub>4</sub><sup>2-</sup>, 1.2; H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 1.2; HCO<sub>3</sub><sup>-</sup>, 25.0 and (+)-glucose, 11.1) at a concentration of 10<sup>6</sup> cells·ml<sup>-1</sup>. Krebs-Henseleit solution was used as the final suspension medium as this was the solution in the organ baths. Neutrophil purities were quantified by performing a differential count on Giemsa stained cytopsin smears. Cell viabilities were assessed by trypan blue exclusion.

Before activation, the neutrophil suspensions were equilibrated in a 37°C shaking water bath for 10 min. 4% w/v human serum albumin (Commonwealth Serum Laboratories) was then added. To activate the neutrophils 1 μM calcium ionophore, A23187 (Sigma, St Louis, MO), was added to half the cell samples and an equal volume of diluent (0.1% ethanol) was added to the remaining cell samples. The neutrophil suspensions were then incubated for a further 10 min. The reaction was stopped by placing it on ice and immediately centrifuging at 10,000 g for 30 s at 4°C. The supernatant was collected, the cells from the activated and nonactivated samples discarded, and the supernatant stored at -70°C until required.

#### *Eosinophil separation and activation*

Normodense eosinophils were purified from the venous blood of healthy volunteers to 88±2% purities and >90% viabilities using a modification of the method of SCHULT *et al.* [13]. After collection into heparin, 5 parts of blood were mixed with 1 part of dextran (Sigma) and the red cells allowed to sediment at room temperature for 40 min. The leucocyte rich plasma was collected and layered onto 3 ml of a percoll solution of density 1.082 g·ml<sup>-1</sup> which had already been layered onto 1 ml of a percoll solution of density 1.1 g·ml<sup>-1</sup>. After centrifugation at 700 g for 20 min the band of cells at the interface of the percoll solutions was collected. These were diluted in HBSS to a concentration of 10<sup>7</sup> cells per ml, layered onto 3 ml of a percoll solution of 1.084 g·ml<sup>-1</sup> and centrifuged at 700 g for 20 min. Remaining red cells were lysed using a buffer of 0.82% NH<sub>4</sub>Cl in 0.1% K<sub>2</sub>CO<sub>3</sub> and the cells washed once in HBSS. The purified eosinophils were resuspended in Krebs-Henseleit solution at a concentration of 10<sup>5</sup> cells per ml. Eosinophil purities and viabilities were quantified as described above.

Eosinophils were activated in the same way as for neutrophils, using 1 μM A23187 after the addition of albumin (4% w/v). As for the neutrophils, a matched set of control samples were prepared in which the A23187 was replaced with diluent (0.1% ethanol). The reaction was stopped after 15 min, the supernatant collected, cells discarded and the supernatant stored at -70°C.

#### *Bronchial tissue*

Human lung was obtained from specimens resected at thoracotomy and transported to the laboratory on ice in Krebs-Henseleit solution that had been saturated with 5% CO<sub>2</sub> in O<sub>2</sub>. Bronchi of 2–3 mm internal diameter were dissected free from the surrounding tissue and stored overnight at 4°C. The next day the bronchi were cut into rings 4–5 mm long. The tissues were suspended in 5 ml siliconized organ baths containing Krebs-Henseleit solution maintained at 37°C and aerated with 5% CO<sub>2</sub> in O<sub>2</sub>. Each tissue was attached to a Grass FTO3 transducer (Grass Instruments, Quincy, MA) and a load of 1 g applied. Changes in tension were measured isometrically and recorded on a Grass 7D polygraph chart recorder. Tissues were allowed to equilibrate over a period of 1–2 h during which the resting tone was continually readjusted to 1 g and the bathing solution changed at 15–20 min intervals. When baseline tension was stable, tissue responsiveness was assessed using one of two methods.

*a) Bolus doses of histamine.* Since it was important to study the transient or short-lived changes in responsiveness which may be induced by the cell supernatant, experiments were conducted with single bolus doses of histamine. We used this protocol rather than a full concentration response curve which requires a considerable time period and thus we may have missed some of these transient effects. A concentration of histamine was selected to give a contraction of the bronchial tissue that was between 20 and 60% of the maximal response to histamine (0.1–10 μM). The dose of histamine was administered, the magnitude of the contraction measured and the tissue washed until tone returned to baseline level. This process was repeated until 3 successive contractions varying by no more than 10% were obtained. Supernatant from 10<sup>6</sup> activated neutrophils or 10<sup>5</sup> activated eosinophils (0.05–0.1 ml) and their appropriate matched controls (supernatant from nonactivated cells) were then defrosted and immediately added to the organ baths. Supernatant from nonactivated neutrophils or eosinophils was added with A23187 so the concentration of A23187 (0.1 μM) was the same in all baths. Baseline tone was observed for 2 min then tissue responsiveness to histamine reassessed.

*b) Electrical field stimulation (EFS).* Bronchial ring preparations were field stimulated with a constant stimulus applied by electrodes attached to custom-made tissue hooks using Grass SD9 stimulators. The stimulation parameters were chosen so that submaximal contractions were obtained (40–100 V, 2–16 Hz, 1 ms for 20 s). Each tissue was stimulated every 8 min until 3 successive contractions, varying by no more than 10%, were obtained. Supernatant from neutrophils or eosinophils was added as described above. Baseline tone was observed for 2 min and tissue responsiveness to EFS reassessed.

### Statistical analysis

In each tissue the average value for the three successive reproducible contractions was calculated and expressed as 100%. The contraction after the addition of supernatant from activated or nonactivated cells was measured and expressed as a percentage of this average value. The mean value for activated and nonactivated cells was calculated within each experiment and a mean and standard error of the mean (SEM) calculated from all experiments. These mean responses after the addition of supernatant from activated or nonactivated cells were compared. Statistical analysis was performed using a paired two-tailed Student's t-test on the values obtained from activated and nonactivated cells.

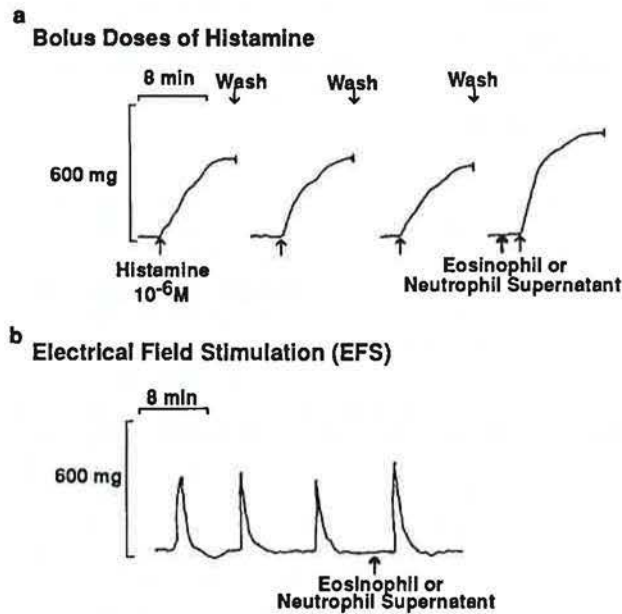


Fig. 1a. - Contractile responses induced by bolus doses of histamine in an individual human bronchus. Addition of histamine was repeated until the contraction height was reproducible. Supernatant from  $10^6$  neutrophils was then added and the histamine response repeated. b. - Contractile responses induced by electrical field stimulation in an individual human bronchus. Stimulation was repeated every 8 min until the induced contractions were stable. Supernatant from  $10^6$  neutrophils was added and then the stimulation repeated.

## Results

### Neutrophils and bronchial tissue

Supernatant from activated neutrophils significantly potentiated the contractile responses of bronchial tissue to both histamine (fig. 1a) and EFS (fig. 1b). The responses to histamine after the addition of supernatant from activated and nonactivated neutrophils were  $153 \pm 15\%$  and  $99 \pm 5\%$  of the control responses respectively (fig. 2) and the difference between these two values was significant ( $p < 0.05$ ,  $n = 5$ ). The contractile responses

of the bronchial tissue to EFS after the addition of supernatant from activated and nonactivated neutrophils were  $111 \pm 3\%$  and  $93 \pm 5\%$  of the control responses, respectively, ( $p < 0.05$ ,  $n = 6$ ) (fig. 2). No contraction of the tissue was observed on addition of neutrophil supernatant.

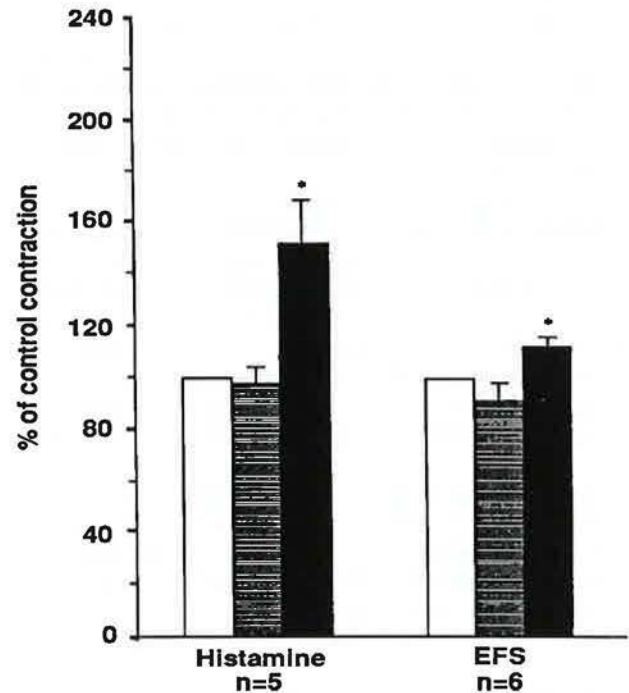


Fig. 2. - The effect of neutrophil supernatant on the responses of human bronchial tissue to histamine and electrical field stimulation (EFS). Vertical bars indicate SEM. \*:  $p < 0.05$ ; □ : control; ▨ : nonactivated neutrophils; ■ : activated neutrophils.

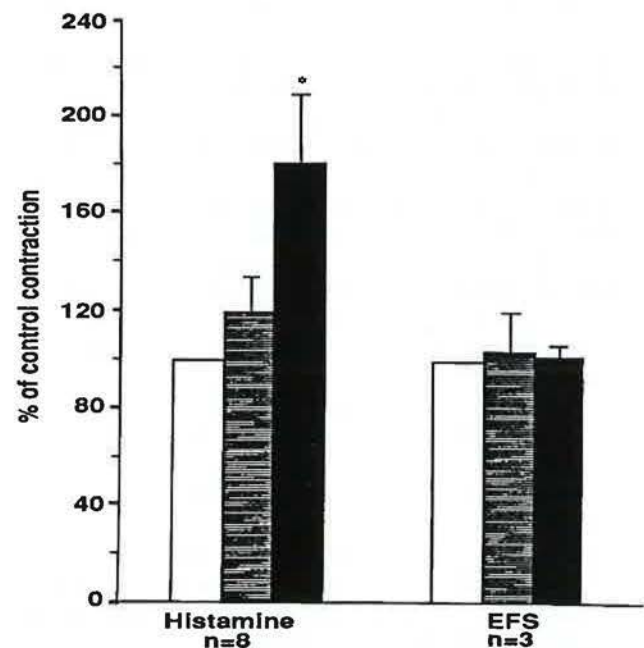


Fig. 3. - The effect of eosinophil supernatant on the responses of human bronchial tissue to histamine and electrical field stimulation (EFS). Vertical bars indicate SEM. \*:  $p < 0.05$ ; □ : control; ▨ : nonactivated eosinophils; ■ : activated eosinophils.

### *Eosinophils and bronchial tissue*

Supernatant from activated eosinophils significantly potentiated the contractile responses of bronchial tissue to histamine but not to EFS (fig. 3). The responses to histamine after the addition of supernatant from activated and nonactivated eosinophils were  $181 \pm 26\%$  and  $121 \pm 12\%$  of the control responses, respectively and the difference between these two values was significant ( $p < 0.05$ ,  $n = 5$ ). Though supernatant from nonactivated eosinophils also appeared to slightly increase tissue responsiveness this was not statistically significant. The responses of the bronchial tissue to EFS after the addition of supernatant from activated and nonactivated eosinophils were  $103 \pm 6\%$  and  $106 \pm 14\%$  of the control responses, respectively ( $p > 0.05$ ,  $n = 3$ ). No contraction of the tissue was observed on addition of the eosinophil supernatant.

### Discussion

This study investigated the possibility that products of neutrophils and eosinophils could increase the responsiveness of human isolated bronchial tissue. Neutrophil products increased the responsiveness of human bronchial tissue to histamine and electrical field stimulation (EFS). Eosinophil products also markedly increased the responsiveness of bronchial tissue to histamine but not to EFS.

A wide range of cell products could be responsible for this increase in responsiveness and identification of these will be the aim of future investigations. A23187 has been shown to cause the release of leukotriene  $B_4$  and platelet activating factor (PAF) from neutrophils [14, 15]. Eosinophils also produce PAF [16] as well as leukotriene  $C_4$  [17] in response to A23187. These products have all been implicated in the development of BHR [18, 19]. Alternatively neutrophils and eosinophils store a variety of proteins and enzymes in cytoplasmic granules which are released into the extracellular environment following cell activation [20, 21]. Major basic protein, from eosinophils, is of particular interest having been shown to increase airway responsiveness by an epithelial dependent mechanism [22]. Further studies will be required to characterise the mediators which increase the responsiveness of human isolated bronchi.

The protocol of the present experiments was designed to detect the effects of short-lived mediators. We therefore chose to study the influence of the supernatant on bolus doses of histamine rather than cumulative concentrations which require a longer time period. In this way we could observe any transient effects of mediators. Although the dose of histamine selected varied, there was no relationship between the magnitude of the contraction and degree of potentiation observed.

The fact that neutrophils increased bronchial tissue responsiveness to EFS while eosinophils did not suggests that products of these cells may be acting on bronchial tissue in different ways. However, it is

possible that eosinophils could increase the EFS response under another set of conditions. Different methods were used to isolate the neutrophils and eosinophils. The incubation times used for cell activation were different. In addition, the number of cells used to generate the eosinophil supernatant was 10-fold fewer than that used for the neutrophil supernatant. This was done to allow the use of eosinophils from healthy donors rather than those from patients with hypereosinophilic syndromes in which eosinophil function may not be normal. An increase in the number of eosinophils may have generated higher concentrations of mediators which may in turn have increased the response to EFS. It is interesting that lower numbers of eosinophils were still able to produce a larger enhancement of the histamine response when compared to neutrophils. However it is difficult to make a direct comparison of the actions of neutrophil and eosinophil products on human isolated bronchial tissue from this study.

The calcium ionophore A23187 was used to activate the cells so as to provide as powerful a cell stimulus as possible without producing a direct contraction of the bronchial tissue [23]. It did not increase bronchial tissue responsiveness when added to control tissues with supernatant from nonactivated cells. Whether a similar increase in responsiveness would be observed if the cells were activated using receptor mediated mechanisms is not known.

Although it is known that BHR is associated with the presence of inflammatory cells, a direct link has not been established in humans. Evidence from animal models is conflicting. In rabbits *in vivo* neutrophils can induce airway hyperresponsiveness [9] but eosinophils do not [11]. In dogs exposure to ozone induces hyperresponsiveness *in vivo* accompanied by increased airway responsiveness *in vitro* [24]. However this *in vitro* hyperresponsiveness may only be transient [2]. In humans, neutrophils and eosinophils have been associated with BHR [6, 25] but it is not known if these cells cause BHR or are simply an associated phenomenon. The present *in vitro* study demonstrates that products from human neutrophils and eosinophils can increase the responsiveness of human bronchial tissue which suggests that they have an active role *in vivo*.

Finally, it is possible that the lack of correlation between the *in vivo* and *in vitro* responsiveness of human bronchial tissue [3-5] was due to the loss of a short-lived mediator made by inflammatory cells. The design of the present study allowed the transient effects of such a mediator to be observed and provides opportunity for further characterisation of the mediators responsible for increasing bronchial responsiveness.

**Acknowledgement:** The authors thank the cardio-thoracic surgeons, theatre staff and pathologists of Royal Prince Alfred, Repatriation General, Royal North Shore and St. Vincent's hospitals for the supply of lung tissue.

## References

1. Boushey HA, Holtzman MJ, Sheller JR, Nadel JA. – Bronchial hyperreactivity. *Am Rev Respir Dis*, 1980, 121, 389–413.
2. Walters EH, O'Byrne PM, Graf PD, Fabbri LM, Nadel JA. – The responsiveness of airway smooth muscle *in vitro* from dogs with airway hyperresponsiveness *in vivo*. *Clin Sci*, 1986, 71, 605–611.
3. Vincenc KS, Black JL, Yan K, Armour CL, Donnelly PD, Woolcock AJ. – Comparison of *in vivo* and *in vitro* responses to histamine in human airways. *Am Rev Respir Dis*, 1983, 128, 875–879.
4. Armour CL, Lazar NM, Schellenberg RR, Taylor SM, Chan N, Hogg JC, Paré PD. – A comparison of *in vivo* and *in vitro* human airway reactivity to histamine. *Am Rev Respir Dis*, 1984, 129, 907–910.
5. Whicker SD, Armour CL, Black JL. – Responsiveness of bronchial smooth muscle from asthmatic patients to relaxant and contractile agonists. *Pulm Pharmacol*, 1988, 1, 25–31.
6. Holtzman MJ, Fabbri LM, O'Byrne PM, Gold BD, Aizawa H, Walters EH, Alpert SE, Nadel JA. – Importance of airway inflammation for hyperresponsiveness induced by ozone. *Am Rev Respir Dis*, 1983, 127, 686–690.
7. Kirby JG, Hargreave FE, Gleich GJ, O'Byrne PM. – Bronchoalveolar cell profiles of asthmatic and nonasthmatic subjects. *Am Rev Respir Dis*, 1987, 136, 379–383.
8. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. – Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. *Am Rev Respir Dis*, 1988, 137, 62–69.
9. Irvin CG, Baltopoulos G, Honour J, Seccombe JF, Henson PM. – Lipid mediators released by activated human neutrophils which increase airways reactivity. *Am Rev Respir Dis*, 1986, 133, A175.
10. Tamaoki J, Sekizawa K, Ueki IF, Graf PD, Nadel JA, Bigby TD. – Effect of macrophage stimulation on parasympathetic airway contraction in dogs. *Eur J Pharmacol*, 1987, 138, 421–425.
11. Uchida DA, Kimani G, Larsen GL, Irvin CG. – Effects on airways function of supernatants from phagocytosing eosinophils. *Am Rev Respir Dis*, 1987, 135, A178.
12. Ferrante A, Thong YH. – A rapid one-step procedure for purification of mononuclear and polymorphonuclear leukocytes from human blood using a modification of the hypaque-ficoll technique. *J Immunol Methods*, 1978, 24, 389–393.
13. Shult PA, Graziano FM, Wallow IH, Busse WW. – Comparison of superoxide generation and luminal dependent chemiluminescence with eosinophils and neutrophils from normal individuals. *J Lab Clin Med*, 1985, 106, 638–645.
14. Walsh CE, Waite BE, Thomas MJ, DeChatelet LR. – Release and metabolism of arachidonic acid in human neutrophils. *J Biol Chem*, 1981, 256, 7228–7234.
15. Ludwig JC, Hoppens CL, McManus LM, Mott GE, Pinckard RN. – Modulation of platelet-activating factor (PAF) synthesis and release from human polymorphonuclear leukocytes (PMN): role of extracellular albumin. *Arch Biochem Biophys*, 1985, 241, 337–347.
16. Lee T, Lenihan DJ, Malone B, Roddy LL, Wasserman SI. – Increased biosynthesis of platelet-activating factor in activated human eosinophils. *J Biol Chem*, 1984, 259, 5526–5530.
17. Weller PF, Lee CW, Foster DW, Corey EJ, Austen KF, Lewis RA. – Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C<sub>4</sub>. *Proc Natl Acad Sci USA*, 1983, 80, 7626–7630.
18. O'Byrne PM, Leikhauf GD, Aizawa H, Bethel RA, Ueki IF, Holtzman MJ, Nadel JA. – Leukotriene B<sub>4</sub> induces airway hyperresponsiveness in dogs. *J Appl Physiol*, 1985, 56, 1941–1946.
19. Barnes PJ. – New concepts in the pathogenesis of bronchial hyperresponsiveness. In: PAF, Platelets and Asthma. M. Schmitz-Schumann ed., Basel, Birkhauser Verlag, pp. 93–101.
20. Weiss SJ. – Tissue destruction by neutrophils. *New Engl J Med*, 1989, 320, 365–375.
21. Gleich GJ, Flavahan NA, Fujisawa T, Vanhoutte PM. – The eosinophil as a mediator of damage to respiratory epithelium: a model for bronchial hyperreactivity. *J Allergy Clin Immunol*, 1988, 81, 776–781.
22. Flavahan NA, Slifman NR, Gleich GJ, Vanhoutte PM. – Human eosinophil major basic protein causes hyperreactivity of respiratory smooth muscle. *Am Rev Respir Dis*, 1988, 138, 685–688.
23. Marthan R, Armour CL, Black JL. – Extracellular calcium and human airway muscle: ionophore A23187 induced contraction. *Respir Physiol*, 1988, 71, 157–168.
24. Jones GL, O'Byrne PM, Pashley M, Serio R, Jury J, Lane CG, Daniel EE. – Airway smooth muscle responsiveness from dogs with airway hyperresponsiveness after O<sub>3</sub> inhalation. *J Appl Physiol*, 1988, 65, 57–64.
25. Seltzer J, Bigby BG, Stulberg M, Holtzman MJ, Nadel JA, Ueki IF, Leikhauf GD, Goetzl EJ, Boushey HA. – O<sub>3</sub>-induced change in bronchial reactivity to methacholine and airway inflammation in humans. *J Appl Physiol*, 1986, 60, 1321–1326.

*Les produits d'origine neutrophilique ou éosinophilique augmentent la réactivité du tissu bronchique humain isolé.* A.R. Hallahan, C.L. Armour, J.L. Black.

RÉSUMÉ: Cette étude a examiné la possibilité que des produits provenant de neutrophiles ou d'éosinophiles augmentent la réactivité du tissu bronchique humain isolé. Des neutrophiles et des éosinophiles ont été isolés du sang périphérique de volontaires sains. Les cellules ont été incubées avec 1 µM calcium ionophore A23187 pendant 10 à 15 minutes, puis centrifugées; le surnageant a été prélevé et conservé à -70°C. Des anneaux bronchiques humains d'un diamètre de 2 à 3 mm et de 3 à 4 mm de long ont été préparés à partir de spécimens réséqués au cours de thoracotomies. Les tissus ont été mis en suspension dans des bains organiques, sous une charge de 1 g, et les modifications de tension ont été mesurées de manière isométrique. Des contractions stables à des doses bolus d'histamine de 0.1 à 10 µM ou à une stimulation par champ électrique (40 à 100 V, 4-16 Hz, 1 ms pendant 20 s) ont été déterminées. Le surnageant provenant de 10<sup>6</sup> neutrophiles ou de 10<sup>5</sup> éosinophiles a été ensuite additionné, et la réactivité tissulaire réappréciée. Le surnageant de neutrophiles augmente la réactivité tissulaire à l'histamine et aux stimulations par champs électriques de 54±17% (n=5, p<0.05) et de 18±7% (n=6, p<0.05) respectivement. Le surnageant d'éosinophiles a augmenté la réponse à l'histamine de 60±23% (n=8, p<0.05), alors que réactivité tissulaire à la stimulation par champ électrique restait inchangée (n=3). Donc, comme les neutrophiles et les éosinophiles peuvent modifier la reactivite de la bronche humaine *in vitro*, il est possible qu'ils aient ce même effet *in vivo*, et qu'ils ne soient pas simplement en relation temporaire avec le développement de l'hyperréactivité bronchique.

*Eur Respir J.*, 1990, 3, 554–558.