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Priming of circulating human eosinophils following late response to allergen challenge

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Priming of circulating human eosinophils following late response to allergen challenge. D.J. Evans, M.A. Lindsay, B.J. O'Connor, P.J. Barnes. ©ERS Journals Ltd 1996.

ABSTRACT: The eosinophil is recognized as an important effector cell in asthma. To investigate the role of eosinophils in the aetiology of asthma, we have examined whether peripheral blood eosinophils are primed following whole-lung allergen challenge.

Nineteen mild asthmatics, who each demonstrated a late asthmatic response (LAR), were studied. The priming of eosinophils following allergen challenge was assessed by measuring: 1) platelet-activating factor (PAF) and complement factor 5a (C5a) stimulated H_2O_2 release upon fibrinogen-coated tissue culture plates (n=8); and 2) *ex-vivo* cell survival (n=11). Subjects were venepunctured before and 24 h after allergen challenge and eosinophils were prepared by immunomagnetic separation.

Basal H_2O_2 release was increased from eosinophils following allergen challenge. The response to PAF stimulation was significantly increased in terms of cell sensitivity (negative log of concentration producing half the maximal effect (pD_2)) and responsiveness (maximum effect E_{max}). With C5a, although there was no change in sensitivity there was a significant increase in the maximal response. *Ex-vivo* eosinophil survival (at 3 days and cell half-life) was significantly prolonged following allergen challenge.

In conclusion, we have demonstrated the priming of circulating eosinophils following allergen challenge.

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Eosinophils play an important role in allergic inflammation [1]. Eosinophil granule proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO), have been implicated in the shedding of airway epithelium, neural stimulation and in the degranulation of mast cells [2, 3]. Eosinophils also release several inflammatory mediators, including platelet-activating factor (PAF) [4], leukotriene C_4 (LTC_4) [5] and superoxide anions following nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase activation [6], which may contribute to airway inflammation. Peripheral blood and sputum eosinophilia are recognized features of asthma, and eosinophil proteins can be detected in the sputum and bronchoalveolar lavage fluid (BALF) of asthmatic patients [7].

Following segmental and whole-lung allergen challenge, there is an early and late asthmatic response. The late asthmatic response (LAR) is characterized by the recruitment of eosinophils into the airway [8]. Comparison of peripheral blood and bronchoalveolar lavage (BAL) suggests that eosinophils undergo activation during their migration into the lung [9, 10]. The mechanism underlying both the recruitment and activation is still unknown, although a number of eosinophil primers, including interleukin-3 (IL-3), interleukin-5 (IL-5), granulocyte-macrophage colony-stimulating factor (GM-CSF) and PAF [11, 12], have been identified at inflammatory lung sites. Up to the present time there have been no reports concerning the effect of allergen challenge upon circulating eosinophils. The release of mediators from the site of inflammation and into the circulation might be expected to prime eosinophils and/or promote their recruitment into the lungs, a process that may be important in the aetiology of the LAR.

Using the release of hydrogen peroxide (H_2O_2) as a measure of NADPH oxidase activation and an *ex-vivo* eosinophil survival assay, we have therefore examined whether peripheral blood eosinophils are primed 24 h following whole-lung allergen challenge.

Materials and methods

Protocol

Nineteen mild asthmatics, demonstrating both an early and late asthmatic response following whole-lung allergen challenge, participated in the study. Eosinophils from eight volunteers were used to measure hydrogen peroxide (H_2O_2) release, whilst eosinophils from 11 volunteers were used in the survival assay. Blood was taken before and 24 h after allergen challenge.

Subjects

All subjects demonstrated clinical features of asthma and had a provocative concentration of histamine causing a 20% fall in forced expiratory volume in one second (FEV_1) (PC_{20} histamine) of less than 4 $mg \cdot mL^{-1}$. All subjects had positive skin-prick tests (>6 mm) to common aeroallergens. The allergen producing the most positive skin response was selected for the allergen challenge (table 1). None of the subjects had previously taken inhaled or oral glucocorticosteroids, and the only medication used was salbutamol on an "as required" basis.

Baseline lung function

At screening, the baseline mean FEV_1 for both groups was 4.2 L (range 3.6–4.5 L). The PC_{20} histamine was measured preallergen and gave a geometric mean of 0.66 $mg \cdot mL^{-1}$ with a geometric range of the SEM 0.55–0.78 $mg \cdot mL^{-1}$ (table 1).

Allergen challenge

The subjects were admitted to the Royal Brompton Hospital Clinical Studies Unit for a 24 hr period. On arrival, they underwent a clinical assessment prior to blood being taken and subsequent allergen challenge. Allergen inhalation was performed using a nebulizer attached to a breath-actuated dosimeter (Dosimeter, MB3; MEFAR Electromedical, Bovezzo, Italy). The nebulizer delivered particles with an aerodynamic mass median diameter of 3.5–4 µm at an output of 9 $\mu L \cdot breath^{-1}$. The nebulizer was set to nebulize for 1 s with a pause time of 6 s at a pressure of 22 psi. Freeze-dried allergen extracts (Aquagen SQ; Allergologisk Laboratium Horsholm, Denmark) were used. Known dilutions of the allergen were made, to give final concentrations of 200, 1,000, 2,500, 5,000, 12,500, 25,000 and 50,000 $IU \cdot mL^{-1}$. The initial dose for the allergen inhalation test was 200 $IU \cdot mL^{-1}$, and FEV_1 was measured 5 and 10 min after each allergen dose. Serially increasing doses of allergen were inhaled and the cumulative dosage resulting in a 15% reduction within 10 min was recorded and constituted an adequate challenge for the early asthmatic response. The FEV_1 was recorded every 15 min for the first hour and hourly thereafter. The LAR was defined as a fall in FEV_1 of 15% from the postsaline FEV_1 between 4 and 10 h. The individual doses of allergen given are presented in table 1. Between 21 and 24 h the subjects were venepunctured for a second time.

Eosinophil preparation

Eosinophils were separated using the method of HANSEL *et al*. [13]. Briefly, venous blood (100 mL) was collected in two 50 mL syringes, each containing 10 mL of acid citrate dextrose anticoagulant. Leucocytes were obtained by sedimenting 5 volumes of blood with one volume of 6% dextran 110 for 45 min at 22°C. The

Table 1. – Patient details

Pt No.	Age yrs	FEV_1 % pred	Allergen dose $IU \cdot mL^{-1}$	EAR Max ΔFEV_1 %	LAR Max ΔFEV_1 %	Blood eosinophil count $10^9 \cdot L^{-1}$	PC_{20} $mg \cdot mL^{-1}$
Volunteers used to measure H_2O_2 release							
1	29	80	31200#	23	17	0.2	0.19
2	23	87	30000‡	27	15	0.4	0.95
3	27	100	1250$	61	35	0.8	0.28
4	29	92	6240‡	35	62	0.2	0.70
5	24	87	3700#	59	16	0.3	0.32
6	29	114	30000‡	28	17	0.2	1.90
7	26	91	6240#	30	19	0.1	0.63
8	21	82	3750#	48	30	0.4	0.19
Volunteers used in the survival assay							
9	25	97	18700#	44	28	0.3	1.11
10	29	90	31200#	40	31	0.1	0.50
11	25	105	1250$	36	56	0.4	0.48
12	29	101	43700‡	59	54	0.2	1.25
13	30	101	18700‡	50	51	0.4	2.47
14	27	92	30000#	25	26	0.2	0.87
15	31	87	6240#	34	19	0.4	0.51
16	24	93	18700$	15	19	0.9	0.98
17	31	75	31200#	16	38	0.3	0.30
18	25	91	1250$	25	25	0.2	0.70
19	26	90	18700‡	15	17	0.4	1.90
Mean	27±	92±	17500	35±	30±	0.3±	0.66+
±SEM	1	2		3	4	0.04	(0.55–0.78)+

+: geometric mean and geometric range of SEM. Pt: patient; FEV_1: forced expiratory volume in one second; EAR: early asthmatic response; LAR: late asthmatic response; PC_{20}: provocative concentration of histamine causing 20% fall in FEV_1; ΔFEV_1: change in FEV_1 from baseline. Allergen used for challenge: #: cat; ‡: grass; $: house dust.

leucocyte-rich plasma was collected and centrifuged at 500×g for 10 min, and the cell pellet was resuspended to 8 mL with the serum. This volume was layered onto 1.082 g·L^{-1} Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged at 1,300×g for 25 min at 22°C. Following centrifugation, the granulocyte layer was collected from the bottom of the tube, and the cell suspension was washed in Hank's balanced salt solution (HBSS). For the H_2O_2 assay, the erythrocytes were lysed in ammonium chloride for 10 min. The cells were then washed in HBSS with 2% foetal calf serum (HBSS/FCS). The pellet was resuspended to a volume of 1 mL with HBSS/FCS and a 10 µL sample was taken for a neutrophil and eosinophil count in a haemocytometer. Eosinophils were purified by removal of the contaminating neutrophils using Macs anti-CD16 magnetic beads (Eurogenetics, Teddington UK), using 1 µL of beads per 10^6 neutrophils. This mixture was allowed to incubate for 30 min at 4°C, and the cells were then resuspended in 40 mL with HBSS/FCS and run across a steel wool column within a magnetic field. The CD16+ cells (*i.e.* neutrophils) were retained on the column and the purified eosinophils were collected in the tube below. The cells were washed in HBSS and finally resuspended in hydroxyethylpiperazine ethanesulphonic acid (HEPES) buffer at a concentration of 10^6 cells·mL^{-1}. Eosinophil purity was greater than 99%

H_2O_2 assay

The production of H_2O_2 was used as a marker of eosinophil respiratory burst. H_2O_2 release was measured during a 1 h incubation in 24-well fibrinogen-coated tissue culture plates (Falcon, Cowley, UK), using a modification of the method of ROOT *et al.* [14]. Tissue culture plate wells were coated with protein by incubation with 0.5 mL of 50 µg fibrinogen in RPMI 1640 at 37°C for 120 min. These were then washed 3 times with 1 mL reaction buffer prior to use. Prewarmed eosinophils (10^5 in 100 µL) were added to 900 µL of prewarmed reaction mixture to give a final concentration of: 10 mM HEPES, 138 mM NaCl, 6 mM KCl, 1 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, 1 mM NaN_3, 1 U·mL^{-1} horseradish peroxidase, 30 U·mL^{-1} superoxide dismutase, 5 µM scopoletin and, where relevant, either complement factor 5a (C5a) (10^{-10} M to 10^{-6} M) or PAF (10^{-10} M to 10^{-5} M). Plates were then incubated at 37°C for 60 min before 800 µL of reaction mixture was withdrawn and scopoletin fluorescence ($\lambda_{excitation}$=350 nm; $\lambda_{emission}$=460 nm; slit widths=5 nm) measured using a Shimadzu RF-5000 recording spectrofluorophotometer (V.A. Howe Ltd., Abingdon, UK). The change in fluorescence from preincubation value allowed the H_2O_2 release to be determined by reference to a standard curve calculated using known amounts of H_2O_2.

Eosinophil survival assay

Freshly separated eosinophils were resuspended in RPMI-1640 (Gibco, Paisley, UK) supplemented with 25 mM HEPES, 32 mM L-glutamine, penicillin-streptomycin 100 IU·mL^{-1} and 100 µg·mL^{-1} and 10% FCS ("supplemented RPMI") at a concentration of 2×10^6 cells·mL^{-1}. Twenty five microlitres of the cell suspension was added to 75 µL of the supplemented RPMI in 96-well cell culture plates (Falcon, Cowley, UK) and cultured at 37°C. Cell viability was assessed by trypan blue exclusion on days 0 (*i.e.* on the day of the cell suspension), 1, 3 and 7. On each occasion, 500 cells were counted.

Drugs and chemicals

PAF (stock solution of 100 mM in ethanol), scopoletin (stock solution of 100 mM in ethanol), C5a (stock solution of 1 mM in 0.1% bovine serum albumin (BSA)), phorbol 12-myristate 13-acetate (PMA) (stock solution of 10 mM dimethyl sulphoxide (DMSO)), horseradish peroxidase (100 U·mL^{-1}), superoxide dismutase (6,000 U·mL^{-1}), HEPES, FCS and HBSS were obtained from Sigma (Poole, UK); dextran 110 (average MW ~110,000) from Fluka BioChemica (Poole, UK): L-glutamine, D-penicillin, streptomycin from Gibco (Paisley, UK); NH_4Cl, $MgSO_4$, $CaCl_2$, glucose and azide from Merck (Lutterworth, UK) and fibrinogen from Calbiochem (Nottingham, UK).

Data analysis

The results of all experiments are expressed as mean±SEM. For the eosinophil survival experiments the comparison between groups of data was made using analysis of variance (ANOVA). Comparison at individual time-points was performed using a Wilcoxon's paired test. For the H_2O_2 assay curve fitting and analysis was performed using PRISM (GraphPad Software. San Diego. USA). The response data (negative log of concentration producing half the maximal effect (pD_2) and maximum effect (Emax)) were also analysed using Wilcoxon's paired test. A p-value of less than 0.05 was considered to be significant.

Results

Response to allergen challenge

Nineteen subjects had a well-defined dual response to allergen inhalation (fig. 1). The mean±SEM maximum percentage change in FEV1 for the early response was -35±3% (range -15 to -61%) and for the late response -30±3% (range -15 to -62%) (table 1). The values quoted represent changes as compared to the post diluent FEV1 (fig. 1).

H_2O_2 release

During initial studies, PAF, C5a and PMA stimulated H_2O_2 release from eosinophils in suspension was examined. However, with the exception of PMA, it was not possible to measure an increase in the activity of the

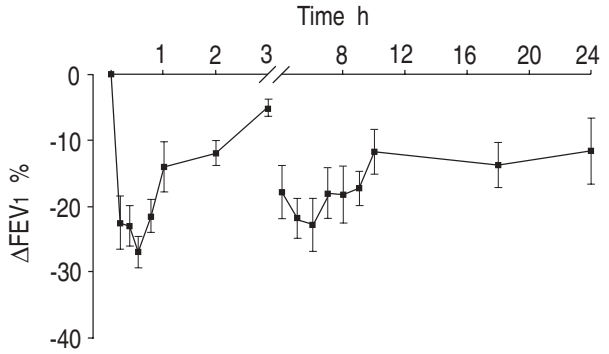

Fig. 1. – Percentage change in forced expiratory volume in one second (FEV_1) plotted against time from baseline after allergen challenge in patients both for early and late asthmatic response. The data are presented as mean±SEM of 19 independent studies. ΔFEV_1: change in FEV_1 from baseline.

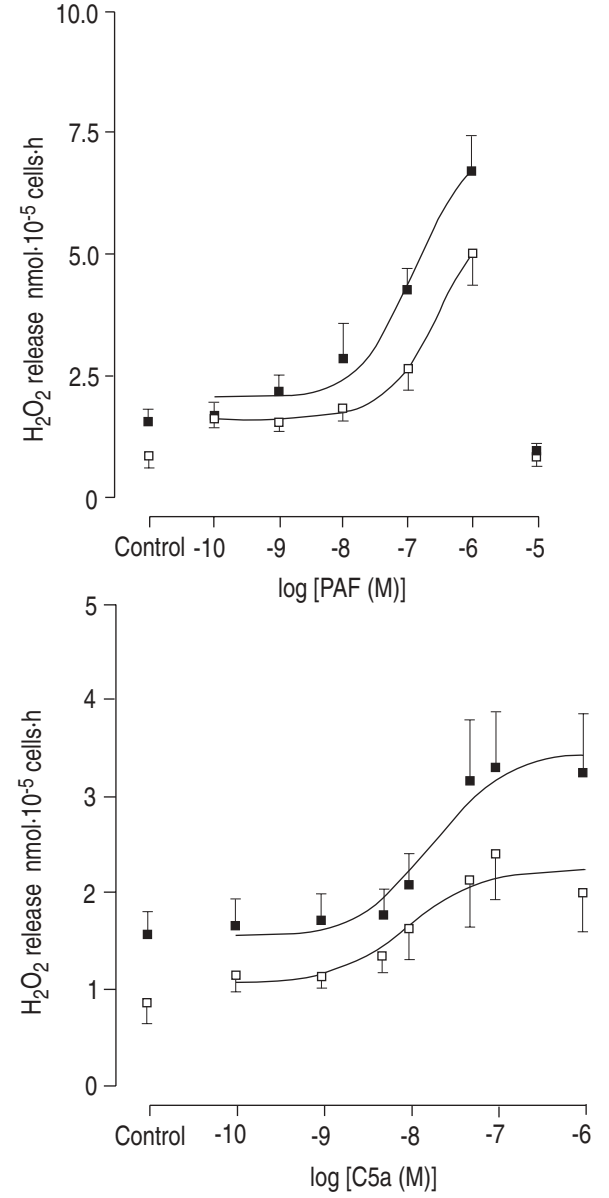

Fig. 2. – Release of H_2O_2 from blood eosinophils stimulated with PAF and C5a in subjects showing a LAR pre- and postallergen challenge. ❑: preallergen; ■: postallergen. The data are presented as mean±SEM of eight independent experiments. PAF: platelet activating factor; C5a: complement factor 5a; LAR: late asthmatic response.

NADPH oxidase (data not shown). Since previous reports had shown activation by a number of soluble factors from eosinophils adherent upon fibrinogen-coated tissue culture plates [15], the H_2O_2 release was investigated under these conditions. Using both C5a and PAF, it was possible to measure a time-dependent release of H_2O_2, which reached a plateau at 60 min.

Following allergen challenge, basal H_2O_2 release from fibrinogen-adherent eosinophils was increased from 0.9±0.2 to 1.6±0.2 nmol $H_2O_2 \cdot 10^{-5}$cells·h ($p<0.05$) (fig. 2). Both C5a and PAF stimulated concentration-dependent H_2O_2 release. In the case of PAF, the E_{max} was assumed to have occurred at 1 µM, since higher concentrations (10^{-5} M) appeared to be toxic to the cells and failed to stimulate a response. This effect was not due to nonspecific inhibition of H_2O_2 release by ethanol, the solvent in which PAF was dissolved, since control experiments showed that higher concentrations of ethanol (0.2% (v/v)) failed to inhibit H_2O_2 generation by 1 µM PAF. There was a significant shift in the dose-response curve to PAF postallergen, with the pD_2 value (-log EC_{50}) changing from 6.8±0.2 preallergen to 7.3±0.3 postallergen ($p<0.05$). The E_{max} for PAF was also increased from 5.0±0.8 nmol $H_2O_2 \cdot 10^{-5}$ cells·h (preallergen) to 6.7±0.7 nmol $H_2O_2 \cdot 10^{-5}$ cells·h (postallergen) ($p<0.05$). For C5a the dose-response curve showed a minimal leftward shift, although this did not achieve statistical significance (pD_2 7.8±0.2 preallergen and 7.9±0.2 postallergen). However, the E_{max} values for C5a, increased from 2.3±0.4 to 3.3±0.6 nmol $H_2O_2 \cdot 10^{-5}$ cells·h preallergen and postallergen, respectively ($p<0.05$) (fig. 2).

Eosinophil survival

Eosinophil survival was increased following allergen challenge (fig. 3). The cell survival at Day 1 was 80% (preallergen) and 86% (postallergen), and on Day 3 the survivals were 59% and 76%, respectively. ANOVA revealed a statistically significant difference between the two sets of data ($p<0.05$). Further analysis showed a significant difference in survival at Days 3 ($p<0.05$) but no difference in survival at the end of the one week incubation period. The count on day 0 reflects cell viability

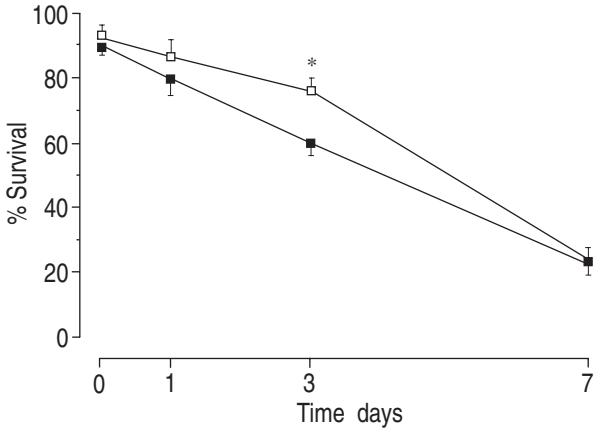

Fig. 3. – *Ex-vivo* survival of eosinophils. ❑: preallergen ; ■: postallergen. *: $p<0.05$, between pre- and postallergen challenge. The data are presented as the mean±SEM of 11 independent experiments.

Table 1. – *Ex-vivo* eosinophil survival

Pt No.	$t_{1/2}$ preallergen days	$t_{1/2}$ postallergen days
1	4.3	7.0
2	5.0	7.0
3	3.0	4.9
4	2.4	4.7
5	2.3	3.1
6	3.9	5.6
7	4.9	5.9
8	3.3	5.2
9	5.3	5.8
10	4.1	4.9
11	4.7	4.9
Mean±SEM	3.9±0.3	5.4±0.3

$t_{1/2}$: 50% cell survival; Pt: patient.

following the separation from whole blood and there was no difference between the pre- and postallergen cells. The eosinophil half-life ($t_{1/2}$) pre- and postallergen was 3.9±0.3 and 5.4±0.3 days, respectively ($p<0.01$) (table 2, fig. 3).

Discussion

We have demonstrated that H_2O_2 release both from unstimulated and PAF-/C5a-stimulated eosinophils was significantly enhanced 24 h following allergen challenge in subjects who show a late asthmatic response (LAR) to allergen. As has been reported previously [15], we were only able to measure this response from eosinophils that were adhered to fibrinogen-coated tissue culture plates, although a number of other investigators have demonstrated NADPH oxidase activation by soluble physiological stimuli from suspended cells [6, 9]. At present, we are unable to explain these contradictory reports. However, from experiments with neutrophils it has been demonstrated that the presence of contaminating lipopolysaccharide during cell separation and subsequent NADPH oxidase activity measurements, has profound effects upon cell function. In experiments where this contamination had been eliminated, adherent but not suspended neutrophils were able to generate O_2^- to soluble physiological stimuli [16].

The mechanism underlying the priming of the NADPH oxidase in circulating eosinophils following allergen challenge is unknown. A recent report has shown that the cross-linking of members of the β_1 and β_2 integrin families triggers eosinophil NADPH oxidase activity [17], whilst in neutrophils, CR3 (β_2 integrin) mediated adherence both "primes" and enhances the oxidase response to soluble stimuli [18, 19]. Since eosinophil activation has been shown to increase the expression both of very late activation antigen-4 (VLA-4) (β_1 integrin) and CR3 (β_2 integrin) [9, 20], this mechanism may explain the observed increase both in basal and PAF-/C5a-stimulated H_2O_2 generation seen in adherent eosinophils following allergen challenge. Interestingly, oxidase activation from suspended eosinophils by the particulate stimuli, opsonized zymosan, is thought to occur through the CR3 receptor by a mechanism that can be primed by prior exposure to PAF [21].

The observed increase in survival times following allergen is less easy to explain and probably involves both *in vivo* priming as well as ongoing influences during the *in vitro* incubation period. Previous work has shown that it is possible to increase eosinophil survival *in vitro* by incubation with the cytokines IL-3, IL-5 and GM-CSF in the presence of 3T3 fibroblasts [22–24]. The observed activation and priming of the NADPH oxidase response, 24 h following allergen, suggest that the eosinophils have been exposed *in vivo* to cytokines generated during the challenge. The increased cell survival by circulating eosinophils is, therefore, probably based on the influence of a number of locally produced soluble mediators. The increased survival *in vitro* incubation is a consequence of *in vivo* priming effects. However, the ongoing production of cytokines (for example IL-5 and GM-CSF) from sources such as Th2 lymphocytes has been removed. Thereafter, there must be additional factors to account for prolonged survival. Firstly, the plethora of mediators to which the eosinophils were exposed during the priming period *in vivo* may have an effect on cellular function that will last for some time after the cells are removed from the circulation. Secondly, eosinophils themselves produce cytokines, such as IL-5 and GM-CSF, and these may be in higher concentration in the culture medium postallergen compared to pre- allergen and, thereby, promote survival.

As with the oxidative response, adhesion may be important both to *in vivo* and *in vitro* survival. Exposure to allergen results in increased expression of adhesion molecules [9, 10]. In these circumstances, increased adhesion to the plastic culture plate wells may increase survival, as has been shown following eosinophils adherence to fibronectin [25]. This binding of the cells may mimic the *in vivo* situation within the tissue.

In summary, we have shown both an increase in basal and PAF/C5a stimulated NADPH oxidase activity from adherent blood eosinophils and prolonged survival in peripheral blood eosinophils of asthmatic patients 24 h following allergen challenge. This is the first report to show *in vivo* priming of circulating eosinophils following exposure to allergen and suggests that localized allergen challenge can produce a systemic response.

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