Priming and activation of airway eosinophils in allergen-challenged guinea pigs and its effect on tracheal smooth muscle reactivity to methacholine and isoprenaline.

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In the present study, the activation state of eosinophils present in the bronchoalveolar lavage (BAL) obtained from actively IgE-sensitised guinea pigs at 6 h or 24 h after saline inhalation (controls) or allergen provocation was explored by assessment of the release of eosinophil peroxidase (EPO) by these cells. In addition, the effect of the BAL cells on guinea pig tracheal smooth muscle tone and reactivity to methacholine and isoprenaline was investigated *in vitro*, using tracheal ring preparations from unchallenged guinea pigs. Both at 6 h (after the early asthmatic reaction; EAR) and at 24 h (after the late asthmatic reaction; LAR) after allergen provocation, the airway reactivity to inhaled histamine *in vivo* was significantly increased, by 2.67 ± 0.74- and 1.66 ± 0.22-fold, respectively (P<0.05 both). In addition, in the BAL of these animals an increased number of infiltrated inflammatory cells, particularly eosinophils, was found. At 6 h and at 24 h after allergen provocation, the basal release of EPO by the BAL eosinophils was significantly increased compared to controls (25.3 ± 2.1 and 5.0 ± 0.6 % of total cellular EPO content, respectively, at 6h (P<0.001), and 37.3 ± 3.7 and 3.2 ± 1.9 %, respectively, at 24h; P<0.001). The total cell content of EPO at 24 h after allergen challenge was, however, significantly lower than that of control as well as of cells obtained at 6 h after the challenge, indicating that these cells were already partially degranulated. Serum-treated zymosan (STZ; 1 mg/ml)-induced EPO release above basal was significantly increased compared to control at 6h after allergen provocation (14.6 ± 2.8 and 3.1 ± 0.6 %, respectively; P<0.05). At 24 h after allergen provocation, the STZ-induced EPO release tended to be enhanced, but was not significantly different from control (7.2 ± 1.9 and 3.6 ± 1.8 %, respectively) and significantly lower than the STZ-induced EPO release at 6 h after allergen provocation (P<0.05). A significant correlation (r = 0.749, P<0.05) was observed between the basal cellular EPO release and airway reactivity to histamine *in vivo* in animals at 6 h after allergen and saline inhalation, while this correlation was absent at 24 h. In addition, the STZ-induced EPO release at 6 h after allergen and saline inhalation was significantly correlated with the number of eosinophils in the airway lumen (r = 0.705, P<0.05). Basal tone of tracheal smooth muscle preparations from unchallenged guinea pigs was significantly increased after 1 h preincubation with BAL cells obtained at 6 h and 24 h after allergen provocation, both in the absence and in the presence of STZ. However, neither the sensitivity (pD₂) nor the maximal responsiveness (E_max) of the tracheal smooth muscle to methacholine and isoprenaline were changed after incubation with STZ-stimulated and non-stimulated BAL cells. The results indicate that allergen provocation causes enhanced airway eosinophilia and activation of these cells to release EPO, both after the EAR and LAR. After the EAR, but not after the LAR, the enhanced activation may contribute to the allergen-induced airway hyperreactivity (AHR) *in vivo*. In addition, enhanced priming of the eosinophils after the EAR may be associated with the infiltration of these cells into the airway lumen. However, activated BAL cells obtained after the EAR, as well as after the LAR, did not directly affect airway smooth muscle function in isolated tracheal ring preparations *in vitro*, indicating that other mechanisms are involved in the allergen-induced AHR after these reactions.
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INTRODUCTION

Allergic asthma is regarded as an inflammatory disease, in which many cell types contribute to its pathophysiology (for review see Borish & Joseph, 1992). In this respect, special attention has been paid to the eosinophil, which is considered to be a major effector cell in the development of allergic asthmatic reactions and in the induction of increased airway responsiveness to non-specific stimuli (De Monchy et al., 1985; Frigas & Gleich, 1986; Beasley et al., 1989; Bousquet et al., 1990). Thus, elevated numbers of activated eosinophils in the airways are associated with the late asthmatic reaction and (allergen-induced) airway hyperreactivity (AHR) to histamine and methacholine (De Monchy et al., 1985; Wardlaw et al., 1988; Beasley et al., 1989; Bradley et al., 1990; Aalbers et al., 1993a,b).

Using eosinophils from healthy donors, it has been demonstrated that eosinophils may be primed and activated by interleukin 5 (IL-5), interleukin 3 (IL-3), granulocyte macrophage colony stimulating factor (GM-CSF) and platelet-activating factor (PAF) to release granular cytotoxic proteins, such as major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN), as well as lipid-derived mediators, such as LTC4 (Bruynzeel et al., 1987; Kroegel et al., 1989; Takafuji et al., 1991; Kita et al., 1992; Blom et al., 1992; Carlson et al., 1993). Primed and activated (hypodense) eosinophils are present in the peripheral blood of patients with allergic asthma (Kauffman et al., 1987; Carlson et al., 1991), and bronchoalveolar lavage (BAL) cells and biopsies taken from the bronchial wall of stable asthmatic patients showed positive staining with EG2, a specific antibody to the secreted form of ECP (Wardlaw et al., 1988; Azzawi et al., 1990), while an increase of EG2-positive cells in these compartments was observed both after the allergen-induced early (EAR) and late (LAR) asthmatic reaction (Aalbers et al., 1993a,b).

Furthermore, MBP has been found in the bronchial wall near sites with shedded epithelium (Frigas et al., 1980) and in the sputum of patients with allergic asthma (Frigas et al., 1981). In guinea pig trachea, MBP, ECP and EPO were shown to induce pathological alterations similar to those found in the airways of asthmatic patients, including exfoliation, bleb formation and ciliostasis towards the epithelium (Motojima et al., 1989). Since the epithelium is an important physical and chemical barrier to inhaled substances (for review see Knight et al., 1994), damage to this barrier may therefore be an important factor in the development of (allergen-induced) airway hyperreactivity (Gleich et al., 1988). In addition, it has been demonstrated that MBP may act as an allosteric antagonist of the autoinhibitory prejunctional muscarinic M2 receptor (Jacoby et al., 1993), which may underlie a muscarinic M2 receptor dysfunction and enhanced vagal reflex activity in asthmatic patients (Minette et al., 1989) and in guinea pigs, both after single (Ten Berge et al., 1995) and repeated (Fryer and Wills-Karp, 1991) allergen challenge.

In a guinea pig model of asthma, we recently demonstrated that the allergen-induced AHR both after the EAR and LAR is associated with enhanced eosinophil numbers and EPO levels in the BAL fluid, indicating that activation of infiltrated eosinophils may be involved in the development of AHR in this model (Santing et al., 1994a;b). In the present study, we investigated the priming and activation state of airway eosinophils in this model.
at 6 and 24 h after allergen challenge (after the EAR and LAR, respectively), by specifically measuring the EPO activity in supernatants from non-stimulated and serum-treated zymosan (STZ)-stimulated BAL cells obtained at these time points. In addition, by \textit{in vitro} incubation of isolated tracheal ring preparations from unchallenged animals with BAL cells from allergen challenged guinea pigs in the absence and presence of STZ, we investigated possible changes in airway smooth muscle reactivity to methacholine and isoprenaline induced by endogenous and exogenous activation of these cells.

\section*{Methods}

\subsection*{Animals}

Outbred specific pathogen free guinea pigs of either sex (Charles River SAVO, Kiszlegg, Germany), weighing 500-800 g, were used in this study. All animals were actively IgE-sensitised to ovalbumin (OA) at three weeks of age as described by Van Amsterdam \textit{et al.} (1989). In short, 0.5 ml of an allergen solution containing 100 \(\mu\)g/ml ovalbumin and 100 mg/ml Al(OH)\(_3\) in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck.

For pleural pressure (\(P_{pl}\)) measurement the animal were operated on in week 3 following sensitisation and used experimentally in week 4 to 8. After the operation, the animals were housed in individual cages, in climate controlled animal quarters and given water and food ad libitum.

All protocols described in this study were approved by the University of Groningen Animal Health Committee.

\subsection*{Measurement of airway function}

Airway function was assessed by on-line measurement of \(P_{pl}\) under unrestrained conditions as described by Santing \textit{et al.} (1992). Briefly, a small latex balloon (HSE, Freiburg, Germany), connected to a saline-filled canula, was surgically implanted inside the thoracic cavity. The free end of the canula was driven subcutaneously to the neck of the animal, where it was exposed and attached permanently. After connection via saline-filled canula to a pressure transducer (TXX-R, Viggo-Spectramed B.V., Bilthoven, The Netherlands), \(P_{pl}\) (in centimetres of H\(_2\)O) was continuously measured, using an on-line computer system. No postoperative inflammation was observed for at least 5 weeks after operation, and baseline \(P_{pl}\) values remained stable during repeated measurements on different days (Santing \textit{et al.}, 1992).

Using a combination of flow measurement with a pneumotachograph, implanted in the trachea, and pressure measurement with the pleural balloon, it was shown that changes in \(P_{pl}\) are linearly related to changes in airway resistance and hence can be used as a sensitive index for histamine- and allergen-induced bronchoconstriction (Santing \textit{et al.}, 1992).
Provocation techniques

Ovalbumin and histamine provocations were performed by inhalation of aerosolised solutions. The provocations were performed in a specially designed animal cage, in which the guinea-pigs could move freely (Santing et al., 1992). The volume of the cage was 9 l, which ensured fast replacement of the air inside the cage with aerosol and vice versa. A De Vilbiss nebulizer (type 646, De Vilbiss, Somerset, PA, USA) driven by an airflow of 8 l/min provided the aerosol required, with an output of 0.33 ml/min.

Histamine provocations were performed starting with a concentration of 25 µg/ml in saline, followed by increasing dosage steps of 25 µg/ml. Each provocation lasted 3 min and provocations were separated by 10-min intervals. The animals were challenged until the $P_{pl}$ increased by more than 100% for at least 3 consecutive min. The provocation concentration causing a 100% increase in $P_{pl}$ ($PC_{100}$) was derived by linear interpolation.

Allergen provocations were performed by inhalation of increasing aerosol concentrations of 1.0, 3.0, 5.0 and 7.0 mg/ml ovalbumin in saline for 3 min, separated by 10-min intervals. Allergen inhalations were discontinued when an increase in $P_{pl}$ of more than 100% was observed. Using these conditions, no anti-histaminic was needed to prevent the development of anaphylactic shock after allergen provocation. All histamine and ovalbumin provocations were preceded by an adaptation period of at least 30 min, followed by two consecutive inhalations with saline solution, lasting 3 min each and separated by a 10-min interval.

Provocation protocol

On two subsequent days before the experimental protocol, the animals were habituated to the provocation conditions. On the first day, the animals were placed inside the provocation cage unconnected to the transducers. Three constructive challenges with saline solution were performed lasting three min each and separated by 10-min intervals. The next day, this protocol was repeated with the animals connected to the measuring system. On the first day of the experimental protocol, baseline histamine $PC_{100}$ was assessed, which was repeated on the next day. Twenty-four hours later, allergen or saline provocation was performed. At 6h after provocation (after the EAR in case of an allergen challenge; Santing et al., 1992; 1994a) and at 24h after provocation (after the LAR), the $PC_{100}$ value for histamine was re-assessed to establish changes in airway reactivity at these time points. Between allergen provocation and the measurement of histamine $PC_{100}$ at 6h, the animals were removed from the provocation cage and placed in a larger cage of 2500 cm$^2$, where they could move around freely and eat and drink ad libitum.

Bronchoalveolar lavage procedure

Animals were anaesthetised with 20 mg/ml Brietal-sodium, 35 mg/kg ketamine hydrochloride and 6 mg/kg Rompun i.p. which ensured a fast, deep anaesthesia. The lungs were gently lavaged via a tracheal canula with 5 ml of sterile saline at 37 °C, followed by
three subsequent aliquots of 8 ml saline. The recovered samples were placed on ice, and centrifuged at 200 g for 10 minutes at 4 °C. The combined pellets were resuspended to a final volume of 1.0 ml in RPMI-1640 medium and total cell numbers were counted in a Bürker-Türk chamber. For cytological examination, cytoplasmic preparations were stained with May-Grünwald and Giemsa. A cell differentiation was performed by counting at least 400 cells in duplicate.

**Eosinophil peroxidase assay**

BAL cells were resuspended in Hanks balanced salt solution (HBSS) to final density of 2.5 \( \times 10^6 \) cells/ml and incubated with medium (control) or STZ (1 mg/ml) for 30 min at 37 °C. Cell stimulation was stopped by placing the samples on ice, followed by immediate centrifugation and subsequent decantation of the supernatant. After decantation the cells were lysed, centrifuged and the supernatant was collected to measure the remaining intracellular EPO content.

The EPO activity in cell supernatants and cell lysates was analysed according to the kinetic assay described by White and colleagues (1991), which is based on the oxidation of O-phenylenediamine (OPD) by EPO in the presence of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)). Substrate was made by dissolving 0.018 % \( \text{H}_2\text{O}_2 \) and 16 mM OPD in 100 mM Tris (hydroxymethyl)-aminomethane-HCl buffer, pH 8.0, containing 0.1 % Triton X-100, immediately prior to use. Horseradish peroxidase (HRP) in increasing concentrations was used as a reference. For the assay, 50 µl of supernatants obtained from either stimulated, non-stimulated or lysed cells or 50 µl of HRP solution were combined with 75 µl of substrate in a polystyrene 96-well microplate and placed into a thermoregulating microplate absorbance spectrophotometer (Thermomax, Molecular Devices, Menlo Park, Ca, USA) at 37°C. Absorbance at 490 nm was measured every 15 sec for 30 min; the velocity of the reaction (as a measure of EPO activity) was calculated by interpolation between 20 successive points (5 min) utilising customised software (Softmax v2.01, Molecular Devices). All samples were assayed in quadruplicate.

Preparation of STZ was as follows: zymosan A in saline (10 mg/ml) was boiled for 1h and, after cooling down, incubated with an equal aliquot of fresh guinea pig serum at 37 °C for 30 min. Subsequently, the STZ was washed twice with fresh saline, the second time overnight in a cold room, to remove any inhibiting factors that may also bind to the zymosan. After this last step, STZ was divided in smaller aliquots and stored at -80 °C.

**Tracheal ring contraction and relaxation**

Non-challenged IgE-sensitised guinea pigs were killed by a sharp blow on the head and exsanguinated. The tracheas were rapidly removed and placed in Krebs-Henseleit (KH) solution (37 °C) of the following composition (mM): NaCl 117.50, KCl 5.60, MgSO\(_4\) 1.18, CaCl\(_2\) 2.50, NaH\(_2\)PO\(_4\) 1.28, NaHCO\(_3\) 25.00, D-glucose 5.50; gassed with 5% CO\(_2\) and 95% O\(_2\); pH 7.4. The tracheas were prepared free of serosal connective tissue and single open ring preparations were mounted for isotonic recording in water-jacketed organ baths.
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(37 °C), containing 20 ml of gassed KH solution, using a 300 mg preload.

After an equilibration period of 30 min, the tracheal ring preparations were relaxed with isoprenaline (0.1 µM) to establish basal tone. The preparations were subsequently washed for 30 min and contracted twice, using 1 and 10 µM, and 0.1, 1, 10 and 100 µM methacholine, respectively, with intermediate washings. Following a washout period of 30 min, the tracheal ring preparations were switched to a 3 ml bath and incubated for 60 min with KH or BAL cells (2 × 10⁶ cells/ml), obtained at 6 or 24h after OA provocation, in the presence or absence of STZ (1 mg/ml). Subsequently, the preparations were washed during 60 min, after which a cumulative concentration response curve (CCRC) with methacholine (either 10 nM to 0.1 mM or 10 nM to 0.3 µM) was made. After reaching a stable tone at 0.1 mM methacholine (maximal contraction) or at 0.3 µM methacholine (half-maximal contraction), a CCRC with (-)-isoprenaline (0.1 nM to 10 µM) was constructed. After the maximal response to the final concentration had been obtained, the preparations were washed twice and 10 µM (-)-isoprenaline was added to establish maximal relaxation.

Data analysis

Changes in the in vivo airway reactivity to histamine induced by allergen provocation were expressed as the ratio of histamine PC₁₀₀ values obtained at 24h before and at 6 or 24h after allergen provocation, respectively (PC₁₀₀ ratio pre/post allergen challenge). EPO activity was expressed as a per cent of total amount of EPO (amount of EPO in the supernatant of stimulated cells + the amount present in the supernatant of the lysed cells). STZ-induced EPO responses were expressed as increase (in % total EPO) above basal. The response of tracheal ring preparations to methacholine was expressed as a percentage of the response obtained by 100 µM methacholine in the second precontraction. For the response of the tracheal preparations to (-)-isoprenaline the maximal relaxation established with 10 µM (-)-isoprenaline, administered after the completion of the CCRC with (-) -isoprenaline, served as maximum response. The sensitivities to these agonists were evaluated as pD₂ (-log EC₅₀) values.

The results are expressed as means ± s.e. mean. Statistical analysis was performed using the Student’s t-test for paired or unpaired observations as appropriate. Correlations were determined using least squares analysis. Differences were considered statistically significant at P<0.05.

Chemicals

Histamine hydrochloride, ovalbumin (grade III), aluminum hydroxide, (-)-isoprenaline hydrochloride, Zymosan A, O-phenylenediamine dihydrochloride, horseradish peroxidase, May-Grünwald and Giemsa stain were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and methacholine chloride from Aldrich (Milwaukee, WI, USA). Brietal-sodium (methohexital) was purchased from Eli Lilly (Amsterdam, the Netherlands), Ketamine hydrochloride from Parke-Davis (Barcelona, Spain), Rompun (2-(2.6-xylidino)-5.6-dihydro-4H-1.3-thiazine-hydrochloride, methylparaben) from Bayer (Leverkusen,
Germany), and RPMI-1640 medium and Hanks balanced salt solution (HBSS) from Gibco Life Technologies (Praisley, Schotland).

**Table 1** Bronchial reactivity to inhaled histamine and eosinophil numbers in the bronchoalveolar lavage (BAL) of sensitized guinea pigs at 6 h and 24 h after saline inhalation or ovalbumin provocation.

<table>
<thead>
<tr>
<th></th>
<th>6h after inhalation</th>
<th>24h after inhalation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>saline</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>BHR (ratio PC&lt;sub&gt;100&lt;/sub&gt; pre/post)</td>
<td>1.04 ± 0.08</td>
<td>2.67 ± 0.74*</td>
</tr>
<tr>
<td>eosinophils/BAL (× 10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>2.6 ± 0.5</td>
<td>6.1 ± 1.0†</td>
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</tbody>
</table>

Results are expressed as the mean ± s.e.mean of 5 to 12 experiments. Statistical analysis: *: P<0.05 compared to before challenge; †: P<0.05 compared to saline; ‡: P<0.05 compared to 6h (Student’s t-test).

**RESULTS**

At 6h as well as 24h after allergen provocation, i.e. after the EAR and LAR, respectively, the airway reactivity to histamine in vivo was significantly enhanced, with a maximal AHR obtained at 6h after the challenge (Table 1). Saline inhalation did not significantly influence bronchial reactivity at both time points (Table 1).

**Figure 1** Basal EPO release of BAL eosinophils obtained from IgE-sensitised guinea pigs at 6h or 24h after either saline or ovalbumin (OA) challenge, expressed as % of total cellular EPO content. 100 % represents 21.9 ± 5.2, 22.5 ± 3.6, 19.3 ± 3.9 and 10.4 ± 1.3 ng EPO/10<sup>7</sup> cells, for 6h saline, 6h OA, 24h saline and 24h OA, respectively. Results are presented as the mean ± s.e.m. of 3-8 animals. Statistical analysis: ***P<0.001 compared to saline; †P<0.05 compared to 6h after OA (unpaired Student’s t-test).

**Figure 2** EPO released from STZ (1 mg/ml)-stimulated BAL eosinophils obtained from IgE-sensitised guinea pigs at 6h or 24h after either saline or ovalbumin (OA) challenge, expressed as % of total cellular EPO content. 100% values are given in the legend to Figure 1. Results are presented as the mean ± s.e.m. of 3-9 animals. Statistical analysis *P<0.05 compared to saline; ‡: P<0.05 compared to 6h after OA (unpaired Student’s t-test).
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Figure 3  Panel A: relationship between STZ-induced EPO release and percentage BAL eosinophils obtained at 6h after saline and allergen provocation. Panel B: relationship between basal EPO release and PC_{100} histamine at 6h after saline and allergen provocation. Least squares analysis: A: r = 0.705, P<0.05; B: r = 0.749, P<0.05.

BAL studies showed a significant increase in the number of airway eosinophils at 6h after allergen provocation compared to saline inhalation. At 24h after allergen provocation, the numbers of eosinophils in the BAL fluid were significantly elevated compared to both saline control and 6h after allergen provocation (Table 1). Figure 1 shows that the basal EPO activity released by the eosinophils was significantly increased at both 6 and 24h after allergen provocation. Moreover, the release, expressed as % of total EPO present in the cells, at 24h was significantly larger than at 6h. However, the total cell content of EPO at 24h after challenge was significantly lower than that of saline controls (10.4 ± 1.3 ng/10^5 cells vs 19.3 ± 3.9 ng/10^5 cells, respectively, P<0.05) as well as that of the 6h cells (22.5 ± 5.2 ng/10^5 cells, P<0.001). Upon stimulation with STZ (1 mg/ml), a significant increase in EPO release from the BAL cells obtained at 6h after allergen provocation was found compared to control cells obtained 6h after saline inhalation (Figure 2). At 24h after allergen provocation, the STZ-induced EPO release still tended to be enhanced, although this was not statistically significant. The stimulated release at this time point was

Table 2  Cellular composition of the bronchoalveolar lavage cells used for incubation with tracheal strips at a concentration of 2 × 10^6 cells/ml. Cells were obtained at 6 and 24 h after ovalbumin provocation.

<table>
<thead>
<tr>
<th>Eosinophils (%)</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Macrophages (%)</th>
<th>Epithelial cells (%)</th>
</tr>
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<tbody>
<tr>
<td>6h after ovalbumin</td>
<td>19.6 ± 6.8 N.S.</td>
<td>6.9 ± 1.8 N.S.</td>
<td>1.1 ± 0.3 N.S.</td>
<td>72.3 ± 8.0 N.S.</td>
</tr>
<tr>
<td>24h after ovalbumin</td>
<td>27.5 ± 4.8 N.S.</td>
<td>12.2 ± 3.0 N.S.</td>
<td>1.8 ± 0.3 N.S.</td>
<td>58.4 ± 5.3 N.S.</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± s.e.mean of 4 to 6 bronchoalveolar lavages. Statistical analysis: N.S.: not significant compared to 6h after ovalbumin (Student’s t-test).
significantly decreased compared to the release at 6h after allergen provocation (Figure 2).

**Table 3** Basal tone of tracheal ring preparations from unchallenged guinea pigs preincubated for 1 h without (Control) or with bronchoalveolar lavage (BAL) cells obtained at 6 or 24 h after ovalbumin challenge, in the absence (Buffer) or presence of 1 mg/ml serum-treated zymosan (STZ).

<table>
<thead>
<tr>
<th></th>
<th>Buffer</th>
<th>STZ</th>
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<tbody>
<tr>
<td>Control</td>
<td>3.1 ± 1.3</td>
<td>3.4 ± 1.6</td>
</tr>
<tr>
<td>BAL cells 6 h</td>
<td>13.9 ± 2.9**</td>
<td>19.2 ± 1.9***</td>
</tr>
<tr>
<td>BAL cells 24 h</td>
<td>20.7 ± 2.7***</td>
<td>31.3 ± 5.8***#</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± s.e.mean of 4 - 9 experiments. Statistical analysis: **: P<0.01; ***P<0.001 compared to Control; #: P<0.05 compared to Buffer (Student's t-test).

**Figure 4** Methacholine cumulative concentration response curves (CCRC) of tracheal ring preparations from unchallenged guinea pigs after 1h incubation in the presence of buffer (circles) or bronchoalveolar lavage cells (2×10⁶ cells/ml; squares) obtained at 6h after ovalbumin challenge (A) or at 24h after challenge (B), in the absence (open symbols) or presence (closed symbols) of 1 mg/ml serum-treated zymosan. Results are means ± s.e.m. of 4-9 experiments.

At 6h after provocation, we observed a significant correlation between STZ-induced EPO release and the number of eosinophils in the BAL (Figure 3A). In addition, there was a significant negative correlation between the basal EPO release at 6h and the PC₁₀₀-value for histamine (Figure 3B). At 24h after provocation, no correlations were found between the above mentioned parameters.
Figure 5  Isoprenaline cumulative concentration response curves (CCRC) of half-maximally contracted tracheal ring preparations in the presence of 0.3 µM methacholine, after 1h incubation in the presence of buffer (circles) or bronchoalveolar lavage cells (2×10⁶ cells/ml; squares) obtained at 6h after ovalbumin challenge (A) or at 24h after challenge (B), in the absence (open symbols) or presence (closed symbols) of 1 mg/ml serum treated zymosan. The tracheae were obtained from non-challenged ovalbumin-sensitised guinea pigs. Results are means ± s.e.m. of 4 to 9 experiments.

Figure 6  Isoprenaline cumulative concentration response curves (CCRC) of maximally contracted tracheal ring preparations in the presence of 0.1 mM methacholine, after 1h incubation in the presence of buffer (circles) or bronchoalveolar lavage cells (2×10⁶ cells/ml; squares) obtained at 6h after ovalbumin challenge (A) or at 24h after challenge (B), in the absence (open symbols) or presence (closed symbols) of 1 mg/ml serum treated zymosan. The tracheae were obtained from non-challenged ovalbumin-sensitised guinea pigs. Results are means ± s.e.m. of 4 to 9 experiments.
In separate experiments, tracheal ring preparations from non-challenged control animals were incubated with BAL cells obtained at 6h or 24h after allergen provocation. The composition of the cell incubates in these experiments is presented in Table 2. Although not statistically significant, the percentage of eosinophils in the 24h cell incubates tended to be higher than that of the 6h suspension. Similarly, the percentages of neutrophils and lymphocytes tended to be higher at 24 h, whereas the percentage of macrophages was decreased. Both in the absence and in the presence of STZ (1 mg/ml) a significant rise of basal tone was observed in tracheal rings incubated with BAL cells obtained at 6h as well as at 24 h after allergen challenge (Table 3). STZ in the absence of BAL cells had no significant effect on tracheal tone on its own. BAL cell-induced increase in basal tone in the absence of STZ tended to be higher with the 24 h cells compared to the cells obtained at 6 h, while this difference was statistically significant in the presence of STZ (Table 3).

After a 1h incubation period with BAL cells, obtained either at 6h or 24h after allergen provocation, in the absence or presence of STZ, no significant changes in both the sensitivity (pD$_2$) and maximal responsiveness (E$_{max}$) of tracheal ring preparations from unchallenged guinea pigs to the contractile agonist methacholine were found (Figure 4; Table 4). Neither was there a significant change in the isoprenaline-induced relaxation of half-maximal or maximal contractile tones induced by 0.3 μM and 100 μM methacholine, respectively (Figures 5 and 6; Table 5).

### Table 4

<table>
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<tr>
<th></th>
<th>E$_{max}$ (%)</th>
<th>pD$_2$ (-logM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>100.9 ± 3.1</td>
<td>6.35 ± 0.13</td>
</tr>
<tr>
<td>+STZ</td>
<td>106.7 ± 3.7</td>
<td>6.51 ± 0.12</td>
</tr>
<tr>
<td>BAL cells 6h</td>
<td>103.1 ± 3.1</td>
<td>6.13 ± 0.23</td>
</tr>
<tr>
<td>+STZ</td>
<td>100.6 ± 4.5</td>
<td>6.25 ± 0.19</td>
</tr>
<tr>
<td>BAL cells 24h</td>
<td>105.7 ± 2.5</td>
<td>6.49 ± 0.17</td>
</tr>
<tr>
<td>+STZ</td>
<td>106.8 ± 2.3</td>
<td>6.29 ± 0.17</td>
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Results are expressed as the means ± s.e.mean of 4 - 9 experiments. E$_{max}$ is expressed as percentage of the contractile response to 100 μM methacholine in the second precontraction (see Methods).

**DISCUSSION**

As previously demonstrated in our guinea pig model of allergic asthma (Santing *et al.*, 1994a), we found a considerable infiltration of eosinophils into the airways at 6 h after allergen challenge (after the EAR), which was further increased at 24 h after the challenge (after the LAR). Both at 6 h and at 24 h after allergen provocation, the eosinophils were activated as indicated by an increase in the spontaneous release of EPO by the BAL cells.
obtained at these time points. These results are fully in line with previous observations of enhanced EPO concentrations in the BAL fluid after the EAR and LAR (Santing et al., 1994b). Activation of infiltrated eosinophils after both the EAR and LAR, as indicated by enhanced immunohistochemical staining with EG2, has also been observed in the BAL as well as in bronchial biopsies of allergic asthmatics (Aalbers et al., 1993a;b). Since cationic proteins released from activated eosinophils are considered to play an important role in the development of AHR (Gleich et al., 1988; Wardlaw et al., 1988; Bradley et al., 1991), the eosinophil activation observed after the EAR and LAR could contribute to the AHR, which is present at these time points, both in guinea pigs (Santing et al., 1994) and in patients (Durham et al., 1988; Aalbers et al., 1991). Indeed, the present study revealed a significant correlation between the spontaneous release of EPO by BAL eosinophils (expressed as % of total cellular content) and the histamine PC<sub>100</sub> at 6 h after allergen challenge. However, such a relationship was absent after the LAR, which could indicate that other mechanisms are more importantly involved in the development of AHR at this time point. It should be noted, however, that the total EPO content of the eosinophils obtained after the LAR was significantly reduced by approximately 50%, which indicates that substantial degranulation had already been taken place and that a direct correlation between the parameters mentioned above may perhaps not be expected.

**Table 5** Maximal effect (E<sub>max</sub>) and potency (pD<sub>2</sub>) of isoprenaline-induced relaxation of tracheal rings from unchallenged guinea pigs, after 1 h incubation without (Control) or with bronchoalveolar lavage (BAL) cells obtained at 6 or 24 h after ovalbumin challenge, in the absence or presence of 1 mg/ml serum-treated zymosan (STZ). Relaxation was measured after contraction of the tracheal rings with 0.3 µM methacholine (approx. 50 % tone) and 100 µM methacholine (approx. 100 % tone).

<table>
<thead>
<tr>
<th></th>
<th>0.3 µM methacholine</th>
<th>100 µM methacholine</th>
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<tr>
<td></td>
<td>E&lt;sub&gt;max&lt;/sub&gt; (%)</td>
<td>pD&lt;sub&gt;2&lt;/sub&gt; (-logM)</td>
</tr>
<tr>
<td>Control</td>
<td>97.5 ± 0.7</td>
<td>8.06 ± 0.11</td>
</tr>
<tr>
<td>+STZ</td>
<td>96.7 ± 0.7</td>
<td>8.08 ± 0.11</td>
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<tr>
<td>BAL cells 6h</td>
<td>95.5 ± 0.9</td>
<td>8.19 ± 0.13</td>
</tr>
<tr>
<td>+STZ</td>
<td>95.8 ± 1.7</td>
<td>8.21 ± 0.18</td>
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<tr>
<td>BAL cells 24h</td>
<td>95.1 ± 3.2</td>
<td>8.20 ± 0.04</td>
</tr>
<tr>
<td>+STZ</td>
<td>97.7 ± 0.8</td>
<td>8.10 ± 0.08</td>
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Results are expressed as means ± s.e.mean of 4 - 9 experiments. E<sub>max</sub> is expressed as percentage inhibition of 0.3 µM or 100 µM methacholine-induced contraction, respectively.

In addition to endogenous activation of the eosinophils after allergen challenge, the cells were also primed to enhanced activation by exogenous STZ. Priming of eosinophils to STZ may be caused by an increased affinity of CR3, a major integrin which acts as a receptor for surface-bound C3b, for STZ (Blom et al., 1992;1994) or up-regulation of CR3 expression (Hartnell et al., 1992) by IL-3, IL-5, PAF and GM-CSF. Although the same cytokines are also involved in direct activation of the eosonophil (Bruynzeel et al., 1987;
Kroegel et al., 1989; Kita et al., 1992; Carlson et al., 1993), a direct correlation between basal EPO release and STZ-induced EPO release after allergen challenge was not observed (not shown), indicating the differential involvement of additional factors. However, at 6 h after challenge there was a significant correlation between the STZ-induced EPO release and eosinophil number in the airways, which may point to a role of the observed priming in the migration of the cells to the airways.

The activated state of inflammatory cells in the BAL obtained at 6 h and at 24 h after allergen challenge was also indicated by an increased basal tone of guinea pig tracheal ring preparations after 1 h incubation with these cells, both in the absence and in the presence of STZ. The cause of this contraction was not investigated, but other studies have indicated that it may be due to enhanced production of LTC$_4$ by activated eosinophils present in the cell suspension (Jongejan et al., 1991). Despite the functional evidence for activation of the BAL cells under the conditions used ($6 \times 10^6$ cells per 3-ml organ bath for 1 h), no changes in methacholine-induced contraction or isoprenaline-induced relaxation of the tracheal rings were observed. In line with these results, Jongejan et al. (1991) demonstrated that isolated blood granulocytes from healthy individuals and atopic subjects induced contraction of human bronchial rings in the presence of STZ, without causing a change in the responsiveness of the bronchial preparations to methacholine at a cell density of $1$ to $5 \times 10^6$ cells per 10-ml organ bath, and with even a reduced sensitivity to the muscarinic agonist in the presence of 10 to $20 \times 10^6$ cells. The cell-induced contraction in that study appeared to be proportional to the number of eosinophils present in the cell incubates, and could be inhibited by the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA), indicating the involvement of eosinophil-derived LTC$_4$ (Jongejan et al., 1991).

By contrast, using BAL cells from repeatedly ovalbumin challenged guinea pigs, a small but significant 30% increase in the maximal contractile response of guinea pig tracheal rings to histamine was recently observed by Van Oosterhout et al. (1996). The enhanced responsiveness could be inhibited by the 5-lipoxygenase inhibitor AA861, also indicating the involvement of leukotrienes. However, in these experiments cellular incubations of the tracheal rings (at a density of $10^6$ BAL cells/ml) were performed during three days.

An effect of activated eosinophils on the contractility of airway smooth muscle ring preparations in vitro may possibly not be expected, since previous studies using human or animal isolated airway strip or ring preparations, demonstrated that (allergen-induced) airway hyperreactivity to contractile agonists in vivo is associated with normal or even decreased reactivity of the $ex vivo$ preparations to these agonists (for review see Meurs and Zaagsma, 1991). This also applies to tracheal ring preparations obtained from our guinea pig model at 6 h and 24 h after allergen challenge (Santing et al., 1994c). By contrast, recently we demonstrated that intact perfused tracheal preparations obtained at 6 and 24 h after allergen challenge did show hyperresponsiveness to histamine and/or methacholine (De Boer et al., 1996; 1998). Since the perfused tracheal preparations retain their structural integrity, the latter observation suggests that allergen-induced airway hyperreactivity is mainly caused by geometric and/or epithelial changes, and not by changes in airway smooth muscle function, which is the major determinant of contraction in airway strip or ring preparations. Indeed, using intact perfused tracheal tube preparations, we have recently demonstrated that a deficiency of nitric oxide (NO), presumably synthesized by constitutive NO synthase (cNOS) present in the airway epithelium, is importantly involved
in the allergen-induced airway hyperreactivity to histamine and methacholine after the EAR (De Boer et al., 1996), while production of NO by inducible NO synthase (iNOS) in the epithelium after the LAR may be involved in the AHR at this time point, presumably by its reaction with superoxide anion to form peroxynitrite (De Boer et al., 1998).

Nevertheless, Omari et al. (1993) demonstrated that 1 h intraluminal exposure of intact bovine bronchial segments with approximately $16 \times 10^6$ guinea pig peritoneal eosinophils, stimulated by the calcium ionophore A23187, did not change the responsiveness of the airways to intraluminally applied acetylcholine, whereas the sensitivity to extraluminal acetylcholine was reduced, similar to the experiments of Jongejan et al. (1991) in the human bronchial ring preparations. By contrast, in an in situ set-up, topical application of fMLP- or PMA-activated human peripheral blood eosinophils on guinea pig tracheal epithelium ($6 \times 10^6$ eosinophils/cm$^2$) induced an enhanced responsiveness of the trachea to intravenously applied acetylcholine after 30 min of incubation, which could be inhibited by a 5-lipoxygenase inhibitor, but appeared unrelated to the release of EPO by the activated eosinophils (Strek et al., 1993). It should, however, be noted that in both of the above mentioned studies heterologous activation systems were used with respect to species origins of cells and airway smooth muscle, while non-asthmatic, non-airway eosinophils were being studied.

In asthmatic patients, as well as in our guinea pig model at 24 h after single and repeated allergen challenge, a reduced $\beta$-adrenoceptor function has been observed in airway smooth muscle strip or ring preparations (Meurs and Zaagsma, 1991; Santing et al., 1994c). The present study indicates that direct interaction between activated eosinophils and the airway smooth muscle is not involved, although inhibition of $\beta$-adrenoceptor radioligand binding by MBP in human lung epithelium has been found (Sneeringer et al., 1993). However, transductional cross-talk between phosphatidyl inositol metabolism, induced by neurotransmitters and (non-eosinophil-derived) mediators such as histamine, and $\beta$-adrenoceptor-induced adenylyl cyclase activation appears to be a more likely mechanism (Meurs and Zaagsma, 1991; Zaagsma et al., 1997).

In conclusion, the present study demonstrates that allergen provocation causes enhanced activation of airway eosinophils to release EPO, both after the EAR and LAR. After the EAR, but not after the LAR, the enhanced activation may contribute to the allergen-induced AHR in vivo, as indicated by a significant correlation between the basal EPO release by BAL eosinophils and the histamine PC$_{100}$ at this time point. However, activated BAL cells obtained after the EAR as well as after the LAR did not directly affect airway smooth muscle function in isolated tracheal ring preparations in vitro, indicating that other mechanisms are involved.

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