

Granulocyte function in the airways of allergen-challenged pigs: effects of inhaled and systemic budesonide

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Summary

Background Late airways obstruction and eosinophil infiltration after allergen challenge are often seen in human asthma and animal models of allergy. This inflammatory reaction, which may be a link between acute and chronic asthma, is blocked by glucocorticoid pretreatment. However, the role of eosinophils in late airways obstruction and the primary site of action of glucocorticoids, i.e. locally or systemically, have not been fully determined.

Objectives This study was initiated to find out the role of eosinophils and neutrophils in allergen-induced late airways obstruction in the pig. The effect of pretreatment with budesonide (BUD) given locally or systemically on cellular responses seen within 8 h after allergen challenge was also studied.

Methods Twenty-five minipigs were actively sensitized with *Ascaris suum* antigen and challenged under anaesthesia with antigen in the lower airways. Pigs were given BUD as an aerosol (10 µg/kg) or an intravenous infusion (5 µg/kg) 1 h before allergen challenge. In one group, high doses of BUD (50 µg/kg) were infused twice with a 3-h interval before allergen challenge. As a positive control, one group was given the BUD vehicle as an infusion and as a negative control, one group not treated with BUD was given the irrelevant antigen ovalbumin. Eosinophils and neutrophils in lung tissue specimens were detected and levels of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) in bronchoalveolar lavage (BAL) fluid were measured using specific antibodies against porcine EPO and MPO.

Results The number of eosinophils in lung tissue and BAL fluid and the level of EPO in BAL fluid were significantly increased 8 h after *A. suum* challenge in pigs not treated with BUD. With regard to possible recruitment and activation of neutrophils the only significant finding was an increase in the number of cells in BAL fluid. The eosinophil numbers and the level of EPO in BAL fluid were shown to be decreased by all BUD treatments in all the compartments studied compared to the positive control. However, the number of eosinophils in lung tissue and EPO levels in BAL fluid did not correlate with the magnitude of the late airways obstruction.

Conclusion Although eosinophils are present in the bronchial wall and lumen and are apparently activated, a causative relationship between this granulocyte and the late bronchial obstruction could not be established in this model.

Keywords: eosinophils, neutrophils, eosinophil peroxidase, myeloperoxidase, immunohistochemistry

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Introduction

Several studies have been performed in humans and in experimental animals to describe the cellular infiltration during allergen-induced late (4–8 h) reactions in the lower airways [1–5]. In most of these models, an eosinophilia and/or signs of eosinophil activation are seen. Neutrophil activation is also sometimes noted, suggesting a general inflammatory response, although eosinophil infiltration seems to predominate. However, the precise cause of the late airways obstruction is unclear. The severity of ongoing asthma seems to correlate with the blood eosinophil number or the activation state of the eosinophil [6, 7], but in dog [2] and primate [4] models, increased numbers of airway neutrophils rather than of eosinophils were seen during late allergic reactions. In nocturnal asthma in man both eosinophil and neutrophil numbers in bronchoalveolar lavage (BAL) fluid have been shown to be increased during the night [8].

We have developed a novel model for studies of late allergic reactions in the pig airways. By controlling endogenous cortisol levels with the cortisol-synthesis inhibitor metyrapone, a late increase in airways resistance and blood eosinophilia could be seen in actively-sensitized pigs 8 h after bronchial challenge with allergen (*Ascaris suum* extract) [9, 10].

Eosinophils and neutrophils participating in the airway inflammation release granule proteins upon activation [11]. Eosinophils have been suggested to specifically release eosinophil peroxidase (EPO) after IgE-stimulation [12]. EPO, alone or together with H_2O_2 and a halide, is toxic to parasites [13] and mammalian cells [14] and can also cause bronchoconstriction as shown in primates [15]. Thus, EPO is potentially able to cause several of the characteristic symptoms of asthma. After activation, neutrophils release myeloperoxidase (MPO) which, together with H_2O_2 and a halide, form an antimicrobial system that is toxic to a wide variety of organisms, e.g. bacteria, fungi and viruses, but also to mammalian cells [16]. For example, both MPO and EPO can, in the peroxidase- H_2O_2 -iodide system, stimulate mast cells to release histamine [17]. For studies of recruitment of eosinophils to lung tissue and their subsequent activation in the pig, polyclonal antibodies and an immunoassay for measurement of porcine EPO in BAL fluid have been developed [18].

Glucocorticoids effectively inhibit the late bronchial obstruction after challenge in humans [19] and the corresponding reaction in animal models [3, 4, 20]. Budesonide (BUD) is a glucocorticoid with very high topical activity; it is not metabolized in the lung, but is rapidly inactivated in the liver [21]. The effects of locally

administered BUD on airway mechanics and mast cell mediator release have recently been studied in the allergic pig [22]. In the present study, the effects of BUD administered by different routes of BUD on the cellular response at 8 h after allergen challenge in anaesthetized minipigs were studied. Eosinophil and neutrophil numbers were determined in peripheral blood, lung tissue specimens and bronchoalveolar lavage. Eosinophils in lung tissue specimens and EPO levels in BAL fluid were detected by the use of antibodies to porcine EPO as previously described [18]. For detection of neutrophils in lung tissue, polyclonal antibodies specific to porcine MPO were developed. These antibodies were also used in the development of a radioimmunoassay for measurement of porcine MPO in BAL fluid.

Material and methods

In vivo experimental procedure

The experiments were approved by the local Ethics Committee for animal research.

Twenty-five male Göttingen minipigs (Ellegaard, Dalmose, Denmark) were used in the study. All of them were sensitized with s.c. injections of a protein fraction purified from an *A. suum* extract [22] in a suspension of $Al(OH)_3$ starting at the age of approximately 6 months. Two booster doses were given at 3-week intervals. At the time of these experiments the pigs weighed 11–23 kg. As previously described [22] the pigs were anaesthetized about 1 week after the last booster injection with a bolus dose of sodium pentobarbitone (Apoteks-bolaget, Umeå, Sweden; 12 mg/kg intravenously in a femoral vein) supplemented with an infusion of 9 mg/kg/h. Pancuronium bromide (Organon, Oss, The Netherlands; 0.2 mg/kg intravenously and subsequently 0.6 mg/kg/h) was given to achieve muscle relaxation. After tracheostomy, the pigs were intubated and ventilated using a Servo ventilator (900; Siemens-Elema, Sweden). All pigs were given a bolus dose of metyrapone (Sigma, St Louis, MO, USA) of 10 mg/kg 2 h before the allergen challenge to normalize plasma cortisol levels [9]. After the bolus dose, a continuous infusion of 1.0 mg/kg/h metyrapone was given.

The body temperature of the pigs was maintained at 37°C during the experiment with a warming pad. Ringer solution was infused intravenously to prevent dehydration. The respiratory pressure was measured by connecting an outlet of the extratracheal tube to a Statham PM 131 TC pressure transducer and this value was used as a measure of transrespiratory pressure, since intrapleural pressure was equal to atmospheric pressure because of a thoracotomy. Airflow was measured with a heated pneumotachygraph (Model 3500A, Hans Rudolph Inc.,

Kansas City, USA) connected to a pressure transducer (Kent Scientific, Litchfield, CT, USA). The signals were sent to an AP 200 Pulmonary Computer (ConMeTech AB, Uppsala, Sweden) for on-line calculations of total lung resistance (R_L) as described elsewhere [22].

BUD (Astra Draco AB, Lund, Sweden) was given to six pigs (the BUD aerosol group) as a dry powder aerosol (mass mean aerodynamic diameter $1.86 \mu\text{m}$, geometric SD 2.0) 1 h prior to allergen challenge as described earlier [22]. The aerosol was directed via the tracheal tube to the lower airways in an amount of $10.2 \pm 1.2 \mu\text{g/kg}$, and the deposition in the airways was estimated to be about 50%. Five pigs were given $5 \mu\text{g/kg}$ BUD as infusion over 5 min 1 h before allergen challenge (BUD infusion group) and three pigs were given infusion of $50 \mu\text{g/kg}$ on two occasions, 3.5 h and 0.5 h before challenge (high dose BUD group). In the vehicle group, six *A. suum* challenged pigs were given an infusion (5 min) of the diluted BUD vehicle (0.4% ethanol and 1.5% polyethylene glycol in saline) 1 h before allergen challenge.

Challenge with the nebulized allergen was performed 4 h after induction of anaesthesia. The allergen consisted of an ultracentrifuged clear solution of an extract of *A. suum* (Pharmacia AB, Diagnostics) with a protein concentration of 7 mg/ml in saline. The allergen (0.5–10 μm) was nebulized in an ultrasonic nebulizer (NB 108, Engström Medical, Stockholm, Sweden) and was delivered via the tracheal tube to the lower airways. Control pigs ($n = 5$) were given nebulized ovalbumin (2 ml, 10 mg/ml) instead of *A. suum* and were not pretreated with BUD.

Arterial blood was collected regularly during 8 h after challenge from a femoral artery and mixed with EDTA to a final concentration of 10 mM. For differential blood leucocyte count, cell smears on glass slides were fixed in methanol, stained with May-Grünwald stain (Merck, Darmstadt, Germany) and then with Giemsa stain (Merck) and 200 cells were counted. Bronchoalveolar lavage (BAL) was performed at 8 h after allergen challenge with 2 aliquots of 50 mL of 37°C saline. The recovered volume was noted, the fluid pooled and centrifuged for 10 min at 4°C and $170 \times g$. The supernatant was stored at -70°C until used for immunoassay. The cell pellet was suspended in 5.0 mL of saline and the cell numbers were counted in Türk's solution (Gentiana violet; Merck, in 6.25 % acetic acid). The cells were diluted in saline to a concentration of $2.0 \cdot 10^5$ cells/mL and 200 μL aliquots were prepared on glass slides using a cytocentrifuge (Cytospin 2, Shandon, Southern Products Ltd, Cheshire, England) spinning at 500 rpm for 5 min. The cytospin preparations were stained with May-Grünwald-Giemsa and 600 cells were counted.

After BAL, the pigs were killed with an overdose of

sodium pentobarbitone and lung biopsy specimens (one per pig) containing parenchymal tissue and bronchioles from the 5th generation (diameter 0.5 to 1.5 mm) and down were taken peripherally from the right middle lobe. The biopsies were snap-frozen in isopentane chilled with dry ice and were stored at -70°C until analysed. BUD was measured at Astra Draco by liquid chromatography and mass spectrometry after extraction as described earlier [22].

Detection of eosinophils in lung tissue

Frozen lung biopsy specimens were cut with a cryostat to a thickness of $6 \mu\text{m}$, transferred to gelatin-cromalum glass slides and stored at -70°C until immunostained. After acetone treatment, the specimens were stained as described previously, using a rabbit polyclonal antibody specific to porcine EPO (RA-EPO) [18]. An alkaline phosphatase-conjugated sheep (Fab) anti-rabbit IgG (Fc γ) (MIAB; Uppsala, Sweden) and Dako Fast Red Substrate System (Dako Corporation, Carpinteria, California, USA) were used for detection. The specimens were counterstained with Mayer's haematoxylin. As a control in all specimens, the primary antibody was replaced with normal rabbit immunoglobulins purified by means of protein A (Pharmacia AB). No staining was observed in these tests. Two sections per lung biopsy specimen, stained with RA-EPO, were coded and analysed in light microscopy. The whole tissue specimen was examined for parenchymal eosinophils at a magnification of $100 \times$ and assigned to one of four classes; class 0 = <5 , class 1 = 5–50, class 2 = 50–200 and class 3 = >200 eosinophils per mm^2 . Eosinophils near the bronchial epithelium were examined at $250 \times$ magnification and eosinophils within $80 \mu\text{m}$ from the epithelium were counted. The mean value of 5 different bronchioles per biopsy specimen was calculated. Photographs were taken with a Zeiss microscope (Axiophot) using Fujicolor 200 ASA. No filter was used.

Polyclonal antibodies against porcine myeloperoxidase

Rabbits were immunized with $50 \mu\text{g}$ porcine MPO (ExOxEmis Inc., Little Rock, Arkansas, USA) in 50% Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA) i.m. in a volume of $500 \mu\text{L}$. Two booster doses with $50 \mu\text{g}$ MPO in 50% Freund's incomplete adjuvant (Difco Laboratories) were given 4 and 8 weeks later. The serum obtained 10 days after the last booster (RA-MPO) was used for immunohistochemistry and in the radioimmunoassay.

Detection of myeloperoxidase-containing cells in lung tissue

MPO-containing cells in lung tissue were detected by immunohistochemical staining using the same method as for EPO. As primary antibody, RA-MPO was used. The antiserum was diluted 1000 times and as a control, serum from an unimmunized rabbit diluted 1000 times was used. The presence of neutrophils was classified in the same way as for eosinophils. Immunocytochemical staining using the same protocol as for lung tissue specimens was also performed on blood smears and cytospin preparations on BAL from pigs with 95% macrophages. Corresponding slides were also stained with May-Grünwald-Giemsa.

Detection of eosinophil peroxidase in bronchoalveolar lavage

Porcine EPO was measured using a sandwich-ELISA described previously [18], using RA-EPO as catching antibody and an alkaline phosphatase-conjugated anti-human EPO mouse monoclonal antibody for detection. The detection limit was 0.15 µg/l.

Detection of myeloperoxidase in bronchoalveolar lavage

All steps were performed in the buffer used as diluent in the Pharmacia eosinophil cationic protein (ECP) radioimmunoassay [23]. RA-MPO antibody was diluted 120 000 times in buffer. The samples, in triplicate unless otherwise stated, and the antibody (50 + 50 µl) were incubated 16 h in tubes at 8°C; pMPO labelled with ¹²⁵I according to the chloramin-T method [24] in a concentration of 10.2 µg/l (specific activity of 0.52 MBq/µg) was added (50 µl) and incubated for 5 h at 8°C. Decanting suspension containing Sepharose anti-rabbit IgG raised in sheep (Pharmacia AB, Diagnostics) was added in a volume of 2 ml and incubated for 30 min at 25°C. The tubes were centrifuged for 10 min at 1500 × g and immediately decanted. The pellet was resuspended in 1.25 ml buffer, centrifuged, and the tubes were decanted once more. The radioactivity was determined using a gamma counter (1260 Multigamma II, LKB, Bromma, Sweden).

Serum from an unimmunized rabbit was used as control. Cross-reactivity was tested against human MPO purified as described earlier [25], human EPO [26] and porcine EPO [27]. The detection limit of the MPO radioimmunoassay was defined as the lowest peroxidase concentration which gave a response 2 standard deviations above the buffer control as determined using 12 replicates. The range of the MPO radioimmunoassay

was determined to be 0.20–50 µg/l. The immunoassay did not cross-react with either human MPO, human EPO or porcine EPO (<0.3%). When adding MPO in a concentration of 1.56 and 12.5 µg/l to BAL fluid, the recovery was 102 and 117 %, respectively. Serial dilutions up to 8 times of two different BAL fluid samples with high MPO concentration resulted in an observed-over-expected value of 100–114%.

Detection of hyaluronan (HA) in bronchoalveolar lavage

Detection of HA in BAL fluid was performed using a radiometric method from Pharmacia AB (Uppsala, Sweden). The test is based on the use of specific HA binding proteins isolated from bovine cartilage. The range of the test was 5–500 µg/l.

Detection of total protein content in bronchoalveolar lavage

Total protein content in BAL fluid was determined by the Pierce BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Company, Rockford, Illinois, USA) using enhanced, colorimetric detection of Cu⁺ produced in the reaction between protein and alkaline Cu²⁺. The range of the test was 10–1200 mg/l. SDS-PAGE on plasma and BAL fluid was performed using PhastSystem (Pharmacia AB) according to the manufacturer and evaluation was made with a GelScan XL (Pharmacia AB).

Statistics and calculations

Mann–Whitney *U*-test was used for comparisons between groups. For correlation analysis the Spearman rank correlation test was used. All evaluations were performed using InStat (GraphPad Software) on an Apple Macintosh computer. Area under the curve was calculated in Excel (Microsoft Corporation, Redmond, WA, USA) using the trapezoid method. Signals were collected and used for the calculation every 6th minute.

Results

In vivo experiments

At 8 h after allergen challenge only very low levels of BUD were found in plasma from the BUD aerosol and BUD infusion group, whereas high levels were found in the high-dose BUD infusion group (Table 1). The levels of BUD in lung tissue at 8 h were relatively high in the high-dose BUD group and BUD aerosol group, but significantly lower in the BUD infusion group (Table 1).

Table 1. BUD levels at 8 h after allergen challenge in plasma and lung tissue*

	BUD infusion (n = 5)	BUD aerosol (n = 6)	High dose BUD (n = 3)
Plasma (nM)	0.16 ± 0.03†	0.16 ± 0.02†	6.5 ± 0.5
Lung tissue (pmol/g dry tissue)	18.4 ± 3.5†	45.2 ± 4.9	53.5 ± 5.5

*Data are presented as mean ± SEM.

†*P* < 0.05 compared to high-dose BUD group (Mann-Whitney *U*-test).

A. suum challenge, but not ovalbumin challenge, caused an acute increase in R_L with a mean peak increase of $158 \pm 51\%$ within 15 min (all *A. suum*-challenged pigs, no difference between groups), which partially resolved within 2 h. The late changes in R_L , calculated as area under the curve between 2 and 8 h after allergen challenge, are presented in Figure 1. In the BUD vehicle group, a late increase in R_L was seen, which was not

blocked by the BUD infusion treatment. However, both BUD aerosol and high-dose BUD infusion inhibited the late increase in R_L .

Blood leucocyte numbers

The arterial levels of white blood cells at the time of allergen challenge (i.e. 1 h 45 min after start of metyrapone infusion) and at 8 h after challenge are presented in Table 2. In a blood sample taken earlier, 30 min after induction of anaesthesia, and representing 'basal' blood cell count, the number of eosinophils and lymphocytes was higher (0.15 ± 0.21 and $6.7 \pm 0.6 \cdot 10^6/\text{ml}$, respectively), and the number of neutrophils lower ($2.6 \pm 0.4 \cdot 10^6/\text{ml}$) compared to the number at the time of allergen challenge. The monocyte and basophil counts were not changed during the surgical preparation.

The eosinophil number started to increase at 4 h after allergen challenge in the BUD vehicle group, at 6 h in the BUD infusion and aerosol groups and at 8 h in the ovalbumin group. Consequently, at 8 h after allergen challenge, a blood eosinophilia was noted in all groups except for the high-dose BUD group, where instead a depletion of eosinophils was seen (Table 2). Also in the BUD aerosol group some inhibition of the eosinophilia was seen compared to the BUD vehicle group. In groups with eosinophilia, the eosinophil differential count was 4–6% of leucocytes at this time point.

A neutrophilia was noted in all the BUD-treated groups (Table 2) and lymphocyte numbers tended to be higher in the BUD aerosol pigs at 8 h compared to the BUD vehicle animals (Table 2). Monocyte numbers were similar in all groups while basophil numbers seemed to be reduced in the pigs with higher levels of circulating BUD (Table 2).

Immunohistochemistry of lung granulocytes

At 8 h, the median value of the lung parenchymal eosinophil score was 1.5 in ovalbumin-challenged pigs.

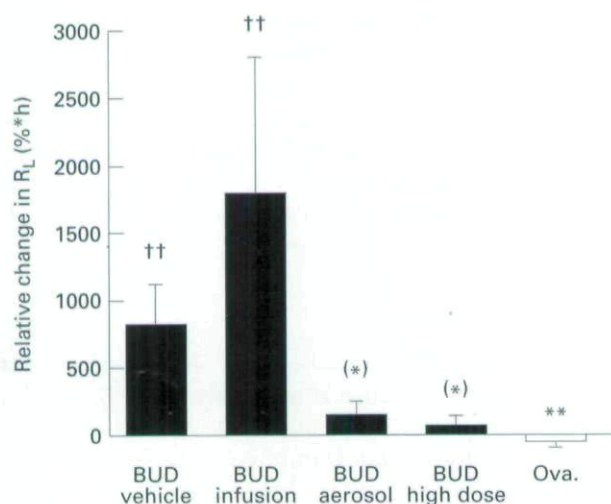


Figure 1. Relative changes in R_L 2 to 8 h after allergen challenge presented as area under the curve. Data are presented as mean and SEM. All pigs were sensitive to *A. suum* antigen. Black bars represent pigs challenged with *A. suum* extract and the white bar represents pigs challenged with a non-relevant antigen (ovalbumin). BUD vehicle: pigs treated with BUD vehicle infusion for 5 min 1 h prior to allergen challenge (*n* = 6). BUD infusion: pigs treated with 5 µg/kg BUD for 5 min 1 h prior to allergen challenge (*n* = 5). BUD aerosol: pigs treated with 10 µg/kg BUD aerosol for 5 min 1 h prior to allergen challenge (*n* = 6). BUD high dose: pigs treated with 50 µg/kg BUD 3.5 h and 30 min prior to allergen challenge (*n* = 3). Ova. = Untreated pigs challenged with ovalbumin (*n* = 5). ††*P* < 0.01 compared to the ovalbumin-challenged group, (**P* < 0.1 and ***P* < 0.01 compared to the BUD-vehicle group (Mann-Whitney *U*-test).

Table 2. Blood cell count ($10^6/\text{ml}$) immediately before and at 8 h after allergen challenge*

		BUD				Ovalbumin challenge (n = 5)
		Vehicle (n = 6)	Infusion (n = 5)	Aerosol (n = 6)	High dose (n = 3)	
Eosinophils	0 h	0.05 $\pm$ 0.02	0.04 $\pm$ 0.03	0.07 $\pm$ 0.02	0.02 $\pm$ 0.02	0.04 $\pm$ 0.03
	8 h	0.61 $\pm$ 0.16	0.56 $\pm$ 0.31	0.29 $\pm$ 0.14	0 $\pm$ 0††	0.54 $\pm$ 0.21
Neutrophils	0 h	4.8 $\pm$ 1.0	9.3 $\pm$ 2.5	8.5 $\pm$ 1.3	6.8 $\pm$ 3.9	4.6 $\pm$ 1.3
	8 h	1.3 $\pm$ 0.26	4.3 $\pm$ 0.96††	4.7 $\pm$ 0.7††	5.9 $\pm$ 1.6††	2.1 $\pm$ 0.7
Lymphocytes	0 h	3.7 $\pm$ 0.9	4.3 $\pm$ 1.2	5.2 $\pm$ 1.0	2.9 $\pm$ 0.9	3.1 $\pm$ 0.8
	8 h	6.9 $\pm$ 1.1	8.0 $\pm$ 2.8	10.1 $\pm$ 1.9	5.0 $\pm$ 0.4	5.6 $\pm$ 1.2
Monocytes	0 h	0.54 $\pm$ 0.23	0.67 $\pm$ 0.19	0.51 $\pm$ 0.12	0.49 $\pm$ 0.30	0.35 $\pm$ 0.13
	8 h	0.21 $\pm$ 0.05	0.35 $\pm$ 0.09	0.29 $\pm$ 0.08	0.31 $\pm$ 0.03	0.23 $\pm$ 0.06
Basophils	0 h	0.06 $\pm$ 0.03	0.03 $\pm$ 0.02	0.05 $\pm$ 0.02	0.01 $\pm$ 0.01	0.12 $\pm$ 0.09
	8 h	0.07 $\pm$ 0.04	0.02 $\pm$ 0.01	0.03 $\pm$ 0.02	0 $\pm$ 0	0.11 $\pm$ 0.06

*Data are presented as mean $\pm$ SEM. † $P < 0.05$ compared to the ovalbumin-challenged group, ‡ $P < 0.05$ and †† $P < 0.01$ compared to BUD vehicle group (Mann-Whitney *U*-test). See legend to Figure 1 for description of the groups. The blood smears were stained with May-Grünwald-Giemsa and 200 cells were counted.

In all *A. suum*-challenged pigs the median score was 2 (non-significant), except in the high-dose BUD pigs (median score 0, $P < 0.05$ compared to the BUD vehicle group). Eosinophils were found close to the epithelium in all *A. suum*-challenged pigs except in the high-dose BUD group (Figure 2a). No significant differences were seen between BUD infusion and BUD aerosol groups. In the BUD infusion pigs (and to some extent also in the BUD vehicle animals), there was a large variation in the number of eosinophils within and between bronchioles (see Figure 3b and 3d). Also large variations between different pigs were noted, resulting in lack of significant differences between these groups and the ovalbumin group. Lower numbers of subepithelial eosinophils were noted in the BUD aerosol pigs, although the number of subepithelial eosinophils was nonetheless elevated in this group compared to the ovalbumin pigs (Figure 2a). In the ovalbumin group only a few eosinophils were detected around the bronchi and there were even fewer in the high-dose BUD group (not shown).

Immunohistochemical staining with RA-MPO and standard May-Grünwald-Giemsa staining labelled the same percentage of the cells in blood smears. The blood cells that were stained using the RA-MPO antibody had a morphology typical of neutrophils (Figure 3f), while the eosinophils with bilobate nucleus were not stained in these preparations. BAL macrophages were not stained with RA-MPO, while BAL neutrophils were stained. High numbers (score 3) of MPO-containing cells were found in the lung parenchyma of all pigs (not shown). There were no significant differences in the number of

MPO-containing cells close to the epithelium in the different groups (Figure 2b).

Bronchoalveolar lavage cells

The recovered volume of BAL fluid was similar in all groups, but the number of recovered cells seemed to be higher in all *A. suum*-challenged pigs compared to the ovalbumin group (Table 3).

The number of eosinophils in BAL fluid was increased in the BUD vehicle group and was suppressed in the BUD-treated groups, and the same pattern was observed for BAL neutrophils (Table 3). Macrophage, lymphocyte and basophil concentrations were not different between the groups except for an increased concentration of lymphocytes in the high-dose BUD group (Table 3). Basophil numbers were low in all groups.

Mediators in bronchoalveolar lavage fluid

Higher concentration of EPO was found in BAL fluid from the BUD vehicle and BUD infusion groups compared to the ovalbumin-challenged group; EPO levels were highly suppressed in the high-dose BUD group and a tendency to suppression was observed in the BUD aerosol group (Figure 4a). The levels of EPO and total protein in BAL fluid did not correlate, and the group differences in EPO levels did not disappear when divided by total protein content. MPO concentration in BAL fluid showed larger variation than EPO levels, but

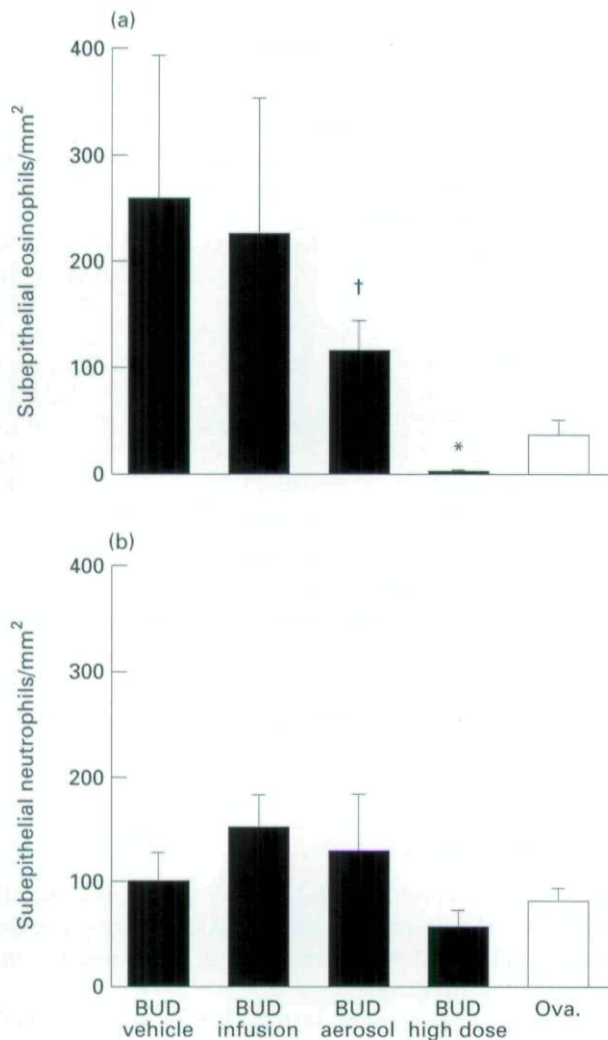


Figure 2. Subepithelial eosinophils (a) (EPO-containing cells) and neutrophils (b) (MPO-containing cells), as revealed by immunohistochemistry. Data are presented as mean and SEM. † $P < 0.05$ compared to the ovalbumin-challenged group and * $P < 0.05$ compared to the BUD-vehicle group, Mann-Whitney *U*-test. See legend to Figure 1 for explanation of groups.

seemed to be increased in all *A. suum*-challenged groups except the high-dose BUD infusion group (Figure 4b).

The concentration of total protein in BAL fluid was not significantly different in any of the five groups (Table 3), although the levels seemed to be higher in the BUD vehicle group compared to the high-dose BUD group. For HA, there was a tendency ($p < 0.1$) towards a higher concentration in the BUD vehicle group compared to the ovalbumin group (Table 3).

When plasma and BAL fluid from one pig with late reaction was run on an SDS-PAGE, a similar protein

pattern was obtained for the two samples, with the most abundant protein being albumin.

Correlation statistics

All *A. suum*-challenged pigs ($n = 20$) are included in the correlation analysis.

The concentration of eosinophils correlated well with EPO in BAL fluid (Table 4) and the concentration of neutrophils correlated well with MPO in BAL fluid (Table 5), but not *vice versa* (not shown). The concentration of MPO and EPO in BAL fluid did not correlate.

The number of blood eosinophils and subepithelial eosinophils correlated, which was not seen for the neutrophils (Table 4 and 5).

The concentration of BUD in plasma showed a positive correlation with blood neutrophil numbers, but not with the number of subepithelial neutrophils in the lung (Table 5). Instead, there was a tendency for BUD levels in plasma and lung tissue to correlate negatively with the number of subepithelial eosinophils (Table 4).

The content of total protein in BAL fluid correlated with BAL fluid HA levels ($P < 0.05$, $r = 0.57$), neutrophil numbers ($P < 0.05$, $r = 0.58$) and MPO levels ($P < 0.01$, $r = 0.64$) but not with BAL eosinophils or EPO.

The magnitude of the late airways obstruction did not correlate with any of the eosinophil-related parameters. The only granulocyte parameter that correlated with late airways obstruction was blood neutrophil numbers.

Discussion

This study was performed in order to characterize the response of the eosinophil and the neutrophil after allergen challenge in the lower airways in the pig. To examine the different effects on cell activation of local and systemical glucocorticoid treatment, BUD was administered as an aerosol in the lung or as an intravenous infusion in low doses chosen to give similar plasma levels.

To make it possible to measure low levels of MPO in the pig, a radio-immunoassay was developed. Polyclonal antibodies specific to porcine MPO were obtained and used in a radioimmunoassay that was found not to cross-react with porcine EPO, human MPO or human EPO. The absence of cross-reactivity is in contrast to rabbit antibodies raised against porcine EPO, which cross-react to a high degree with human EPO [18]. The low detection limit of the porcine MPO radioimmunoassay ($0.2 \mu\text{g/l}$), necessary for detection of neutrophil activation in BAL fluid in this model, was achieved by using sequential incubation. Furthermore, a washing step was introduced

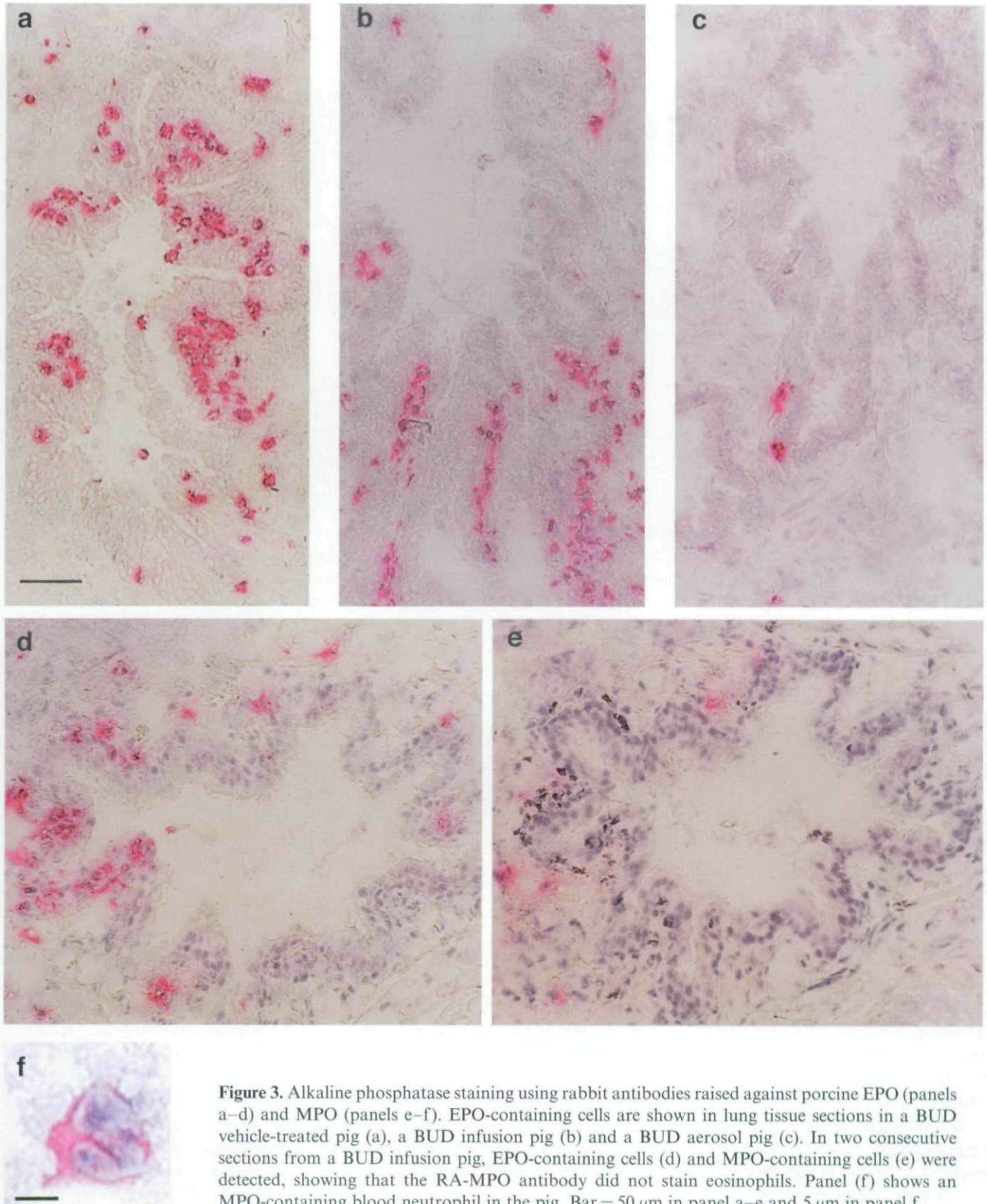


Figure 3. Alkaline phosphatase staining using rabbit antibodies raised against porcine EPO (panels a–d) and MPO (panels e–f). EPO-containing cells are shown in lung tissue sections in a BUD vehicle-treated pig (a), a BUD infusion pig (b) and a BUD aerosol pig (c). In two consecutive sections from a BUD infusion pig, EPO-containing cells (d) and MPO-containing cells (e) were detected, showing that the RA-MPO antibody did not stain eosinophils. Panel (f) shows an MPO-containing blood neutrophil in the pig. Bar = 50 μ m in panel a–e and 5 μ m in panel f.

Table 3. Recovery of BAL fluid and BAL fluid concentration of cells, total protein and hyaluronic acid*

	BUD				Ovalbumin challenge (n = 5)
	Vehicle (n = 6)	Infusion (n = 5)	Aerosol (n = 6)	High dose (n = 3)	
Fluid (ml)	38.5 ± 9.4	53.2 ± 12.0	58.7 ± 8.0	35.7 ± 5.5	46.0 ± 13.6
Total cells (10 ⁴ /ml)	61 ± 6††	40 ± 12	81 ± 21	60 ± 23	26 ± 7††
Macrophages (10 ⁴ /ml)	42.4 ± 9.8	27.8 ± 7.2	68.3 ± 20.6	52.5 ± 22.3	21.1 ± 7.8
Eosinophils (10 ⁴ /ml)	0.71 ± 0.26††	0.07 ± 0.05‡	0.22 ± 0.07	0 ± 0‡	0.04 ± 0.03‡†
Neutrophils (10 ⁴ /ml)	11.8 ± 5.9†	5.6 ± 4.9	7.6 ± 3.1	0.4 ± 0.3‡	1.0 ± 0.5‡
Lymphocytes (10 ⁴ /ml)	5.8 ± 1.4	3.0 ± 0.7	4.8 ± 1.6	7.2 ± 1.3†	3.3 ± 0.8
Metachromatic cells (10 ⁴ /ml)	0.08 ± 0.04	0.06 ± 0.03	0.07 ± 0.06	0.12 ± 0.10	0.09 ± 0.07
Protein (mg/ml)	0.95 ± 0.55	0.87 ± 0.40	0.69 ± 0.28	0.48 ± 0.22	0.60 ± 0.30
HA (µg/ml)	133.4 ± 40.2	73.7 ± 37.3	56.3 ± 21.1	65.8 ± 11.5	45.1 ± 18.0

*Data are presented as mean ± SEM. The blood smears were stained with May-Grünwald-Giemsa and 200 cells were counted.

† $P < 0.05$ and †† $P < 0.01$ compared to the ovalbumin-challenged group, and ‡ $P < 0.05$ and ‡‡ $P < 0.01$ compared to the BUD vehicle group (Mann-Whitney *U*-test). See legend to Figure 1 for description of groups.

to limit the background, probably caused by the basic molecule MPO adhering to the sheep antibody-coupled Sepharose. It has been shown that macrophages in the proximal airways can internalize MPO [28], but as judged by examination in a light microscope, our polyclonal antibody to porcine MPO, which was shown to stain blood and BAL neutrophils, did not stain BAL macrophages. Monocytes have also been shown to contain low amounts of MPO [29], but this will probably not influence the MPO measurements in BAL to any major extent.

A mild eosinophilia was seen in the blood of all groups of pigs at 8 h, including the ovalbumin-challenged pigs, although the eosinophilia appeared later in this group. Like the other pigs, the ovalbumin-challenged pigs were actively-sensitized to *A. suum* extract. This may have triggered eosinopoiesis which could result in eosinophilia when the endogenous cortisol levels were lowered with metyrapone. The eosinophilia was completely blocked at 8 h by high systemic levels of BUD. Eosinophils in lung parenchyma could be noted in all groups, probably reflecting the number of eosinophils in blood. We were not able to determine the exact location (pulmonary capillary or alveolar space) of the parenchymal eosinophils in this study.

According to Vilpo and coworkers [30], 5–50 eosinophils per mm² (corresponding to score 1 in the present study) are found in normal pig lungs. The correlation between the numbers of eosinophils close to the bronchial epithelium and eosinophils in blood indicates active recruitment via the bronchial circulation as earlier sug-

gested by Aalbers and coworkers [31]. The normal numbers of eosinophils in pig bronchus have been shown to be below 20 per mm² [30], suggesting the presence of at least 10 times more eosinophils in and around bronchi in pigs that show late allergic reactions. However, the number of subepithelial eosinophils did not correlate with the magnitude of the late bronchial obstruction.

Subepithelial eosinophils found in the pigs treated with BUD aerosol were evenly distributed, and small intra- and inter-bronchial variations were found. This suggests a uniform distribution of BUD particles to the lower airways, effectively inhibiting local inflammation. The absence of eosinophils in blood, lung tissue and BAL in pigs treated with high BUD doses much resembles the findings in pigs with high cortisol levels [10, 18]. BAL eosinophil numbers were significantly elevated only in BUD vehicle pigs, while the EPO concentration in BAL fluid seemed to be elevated in all *A. suum*-challenged pigs except those treated with high doses of BUD. In the *A. suum*-challenged groups, except in the high BUD infusion group, subepithelial eosinophil numbers seemed to be increased. This implies that EPO in BAL fluid may be derived both from eosinophils in BAL and in tissue.

While high doses of BUD, given systemically, completely blocked eosinophil recruitment and activation at 8 h, low doses of systemic BUD did not have marked effects on eosinophil number in blood or in parenchyma, but the migration of the eosinophils to the airway lumen and the activation of the eosinophils as measured by released

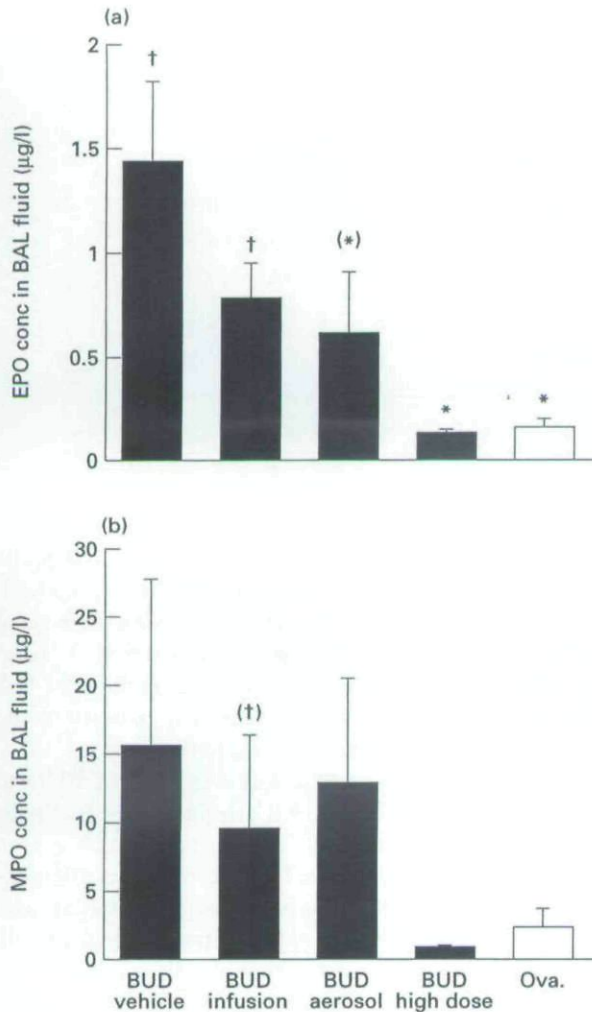


Figure 4. Concentration of EPO (a) and MPO (b) in BAL fluid collected 8 h after allergen challenge. Data are presented as mean and SEM. (†) $P < 0.1$ and ‡ $P < 0.05$ compared to the ovalbumin-challenged group and (*) $P < 0.1$ and * $P < 0.05$ compared to the BUD-vehicle group, Mann-Whitney *U*-test. See legend to Figure 1 for explanation of groups.

EPO was reduced. Since no measurements were done after 8 h after allergen challenge, there is a possibility that the high dose of BUD and the BUD aerosol merely have delayed a late bronchial obstruction. However, no significant correlation between the late increase in R_L and any of the eosinophil-derived parameters was found, suggesting that, within 8 h, the late airways obstruction is not primarily caused by eosinophil activation.

In contrast to eosinophils, the neutrophil number in peripheral blood increased with increasing systemic BUD levels. In lung parenchyma (pulmonary capillaries and alveolar space), the neutrophil was abundant in all groups. There was no significant difference between the groups with regard to the numbers of subepithelial neutrophils, but in BAL fluid from the BUD vehicle group the number of neutrophils was increased. High tissue concentration of BUD decreased the number of neutrophils in BAL, but only the high dose of systemic BUD inhibited the activation of the neutrophil as measured by MPO concentration in BAL fluid.

The late increase in R_L correlated negatively with the number of blood neutrophils, reflecting that BUD both inhibited the late obstruction and increased the number of blood neutrophils. Since the number of blood neutrophils did not correlate with the number of subepithelial neutrophils and since the subepithelial neutrophils did not correlate with neutrophils in BAL fluid, it is suggested that the bronchial circulation is not the primary route for the neutrophil to enter the airway lumen. However, it has been shown that neutrophils enter the alveolar septum and airspace rapidly (less than 2 h) after an inflammatory stimulus in rabbit lungs, and that the passage of cells takes place mainly in the pulmonary capillary bed [32]. The neutrophils entering the alveolar space could then be propelled up to the bronchial mucosa by the mucociliary escalator.

The late increase in R_L could at least partly be due to an increased secretion from submucous glands and/or

Table 4. Correlation statistics for eosinophil parameters*

	Blood eosinophils	Subepithelial eosinophils	Eosinophils in BAL	EPO conc. in BAL
Blood eosinophils	—	0.63††	0.38	0.55†
Lung subepithelial eosinophils	0.63††	—	0.41	0.36
Eosinophils in BAL	0.38	0.41	—	0.82†††
BUD conc. in plasma	-0.45	-0.54	-0.06	-0.03
BUD conc. in lung tissue	-0.34	-0.53	-0.25	-0.25
Late increase in R_L	0.21	0.14	0.31	0.31

*r values using Spearman rank correlation test are presented for all *A. suum*-challenged pigs ($n = 20$).

† $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$. For correlations with BUD concentration, $n = 10-12$.

Table 5. Correlation statistics for neutrophil parameters*

	Blood neutrophils	Subepithelial neutrophils	Neutrophils in BAL	MPO conc. in BAL
Blood neutrophils	—	0.09	-0.12	0.03
Lung subepithelial neutrophils	0.09	—	0.09	0.22
Neutrophils in BAL	-0.12	0.09	—	0.77†††
BUD conc. in plasma	0.77††	-0.19	-0.04	-0.25
BUD conc. in lung tissue	0.15	-0.19	-0.14	-0.22
Late increase in lung resistance	-0.57†	-0.08	0.24	-0.06

**r* values using Spearman rank correlation test are presented for all *A. suum*-challenged pigs (*n* = 20).

†*P* < 0.05, ††*P* < 0.01 and †††*P* < 0.001. For correlations with BUD concentration, *n* = 10–12.

plasma leakage from venules. Characterization of the protein content in BAL fluid from one pig on SDS-PAGE revealed a protein pattern similar to that in plasma, with albumin as the most abundant protein. In this study, the total protein concentration in BAL fluid was used as a measure of plasma leakage. However, it has been suggested that albumin is continuously transferred into the bronchial lumen during the lavage procedure [33], and may therefore not be a good measure of plasma leakage. As a matter of fact, we did not see any significant difference in total protein concentration in BAL fluid between the groups. To achieve a sensitive measure of plasma leakage, larger marker molecules such as fibrinogen [34] or α_2 -macroglobulin [35] should probably be used. Nevertheless, since EPO concentration did not correlate with total protein concentration in BAL fluid, but did correlate with BAL eosinophil numbers, EPO in BAL fluid is probably locally released and not passively transferred from the circulation as a result of leaky vessels. In contrast, both BAL neutrophil numbers and MPO concentration correlated with total protein content in BAL fluid. This indicates that the presence of neutrophils and the neutrophil marker MPO was related to plasma leakage, possibly indicating a change in the alveolar septa resulting in increased neutrophil emigration to the alveoli.

HA levels in BAL fluid have been shown to correlate both with the severity of asthma and with ECP levels in BAL fluid [36]. Since HA is a negatively charged molecule and could bind to cationic proteins (e.g. EPO) it was of interest to measure levels of HA in BAL fluid in this study. We observed a tendency towards higher HA concentration in BAL fluid from the BUD vehicle group compared to the ovalbumin-challenged pigs. Earlier studies have shown an increase in HA in BAL fluid 6 weeks after irradiation lung injury [37] and at 24 h after histamine inhalation [38]. The rapid (< 8 h) appearance

of HA noted in the present study suggests that HA is not derived from activated fibroblasts, but that it may be released from seromucous cells in tracheobronchial submucosal glands [39]. Alternatively, the sudden increase in HA levels may reflect leakage of HA-rich interstitial fluid into the lumen. Theoretically, this increase of HA in the bronchial lumen may decrease the toxicity to the epithelium of the released cationic peroxidases.

It has recently been shown that the levels of BUD in lung tissue, but not plasma, 8 h after allergen challenge correlated negatively with the late bronchial obstruction in the pig [22]. This suggests that the late allergen-induced bronchial obstruction is locally mediated, and is probably initiated by the activation of a resident cell, e.g. the macrophage or the epithelial cell.

In conclusion, this study has shown recruitment and activation of eosinophils, and to some extent of neutrophils, in the late allergic reaction in the lower airways in the pig. Both eosinophil recruitment and the late bronchial obstruction were blocked or at least delayed with high doses of BUD. However, low doses of locally administered BUD, which effectively inhibited the late bronchial obstruction, had only moderate effects on eosinophil recruitment and activation. We conclude that the eosinophils are activated in the late allergic reaction in the lower airways, but a causative relationship between the eosinophils and the late bronchial obstruction could not be established in this model.

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