

# Influence of endogenous cortisol on eosinophil function in sensitized pigs: direct measurements of eosinophil peroxidase

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## Summary

**Background and Objectives** Eosinophils are associated with bronchial asthma, but the role of the eosinophil is not fully understood. This study was initiated in order to study the influence of endogenous cortisol on eosinophil recruitment and activation in allergic inflammation in the lower airways in the pig.

**Methods** Polyclonal and monoclonal antibodies against porcine eosinophil peroxidase (EPO) were raised. Detection of eosinophils in blood smears and lung biopsy specimens was achieved using the polyclonal antibody. For determination of porcine EPO in bronchoalveolar lavage (BAL) fluid, a sandwich enzyme-linked immunosorbent assay (ELISA) with a detection limit of 0.15 µg/L was developed. No cross-reactivity with porcine myeloperoxidase was found. Pigs that had been actively sensitized with repeated subcutaneous injections of *Ascaris suum* antigen were acutely challenged with antigen in the lower airways under pentobarbitone anaesthesia.

**Results** Control animals with plasma cortisol levels of approximately 400 nM did not exhibit infiltration of eosinophils into lung parenchyma or EPO-release in the bronchial lumen within 8 h after challenge. However, in pigs treated with a cortisol-synthesis inhibitor (metyrapone), resulting in plasma cortisol levels of approximately 40 nM, there was a marked eosinophil infiltration into lung tissue at 8 h. Furthermore, EPO levels in BAL fluid were increased in some, although not all, low-cortisol animals. There was no infiltration of eosinophils into skin tissue in these animals.

**Conclusions** It is concluded that, after allergen challenge in the lower airways of metyrapone-treated pigs, newly recruited eosinophils infiltrate lung tissue specifically. Furthermore, a cortisol-sensitive release of the eosinophil-derived cationic protein EPO, into the bronchial lumen was established. This is, to our knowledge, the first description of direct measurements of the release of an eosinophil granule protein in a large animal model of allergy.

**Keywords:** airways, bronchoalveolar lavage, bronchial provocation test, cortisol, immunoassay, immunohistochemistry, metyrapone

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## Introduction

Late asthmatic reactions are seen in human asthmatics

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[1] as well as in animal models of allergy [2], and are thought to reflect major parts of chronic airway inflammation in asthmatics. However, the exact mechanisms involved in the late-phase airways obstruction are unclear. In order to study these mechanisms, a novel model using the pig has been developed [3,4], in which

actively-sensitized pigs receive allergen (*Ascaris suum* extract) in the lower airways. If cortisol-synthesis is inhibited (e.g. with metyrapone) these allergen-challenged pigs exhibit a late (4–8 h) bronchial obstruction, and a blood eosinophilia is also seen starting 4–5 h after allergen challenge. The presence of eosinophils in lung biopsy specimens and bronchoalveolar lavage (BAL) fluid is a common feature in asthmatics [5]. To study the infiltration of eosinophils in lung tissue, immunological staining techniques are valuable. In humans, antibodies against eosinophil cationic protein (ECP) have been used for studies of tissue eosinophils [6], but few reagents for staining eosinophils in experimental animals are available.

To study the function of eosinophils in allergen-induced late-phase reactions in the pig airways, it may be important to determine the degree of activation of the eosinophil. The presence of eosinophils has previously been estimated in animal models using peroxidase activity assays in cell suspensions [7,8], but these enzyme-activity methods are probably not sensitive enough to detect extracellular peroxidase. Of the cationic proteins released by activated eosinophils in man, eosinophil peroxidase (EPO) has been suggested to be the protein most specific to eosinophils [9], and also to be selectively released by IgE-dependent mechanisms [10,11]. Thus, immunological detection of EPO should be a specific and sensitive method for determination of eosinophil number and activity.

In this study we describe methods for immunohistochemical detection of eosinophils in porcine lung biopsy specimens as well as direct measurements of porcine EPO (pEPO) in BAL fluid, using newly developed polyclonal and monoclonal antibodies. By using these methods, the influence of metyrapone pretreatment on eosinophil function was studied after bronchial allergen challenge in actively-sensitized pigs.

## Materials and methods

### Peroxidases

Human EPO (hEPO) and human myeloperoxidase (hMPO) was purified as previously described [12,13]. Porcine MPO (pMPO) was obtained from ExOxEmis Inc., Little Rock, Arkansas, USA and pEPO was purified as described elsewhere from an eosinophil-rich leucocyte fraction derived from pigs infested with *Ascaris suum* eggs.

### Conjugates

Sheep-anti-mouse-IgG-alkaline phosphatase, Sigma

A-5781 (SAM-IgG-ALP) crossreacting with mouse IgA and IgM isotypes was used to detect all monoclonal antibodies. Sheep (Fab)-anti-rabbit-IgG (Fc $\gamma$ )-ALP (SAR-IgG-ALP), Ab 701 (MIAB; Uppsala, Sweden) was used to detect polyclonal antibodies.

### Buffers used in enzyme-linked immunosorbent assay (ELISA)

*Coating buffer* 0.2 M phosphate buffer, pH 9.

*Postcoating buffer* 50 mM phosphate, 0.5% fetal calf serum (FCS, Flow, Solna, Sweden), 0.33 M sorbitol (Merck, Darmstadt, Germany), 0.15% Kathone (Vendico Chemical, Malmö, Sweden), pH 7.0.

*Reagent buffer* 50 mM phosphate buffered saline (PBS), 0.1% Tween 20 (Hefti, Zürich, Switzerland), 0.2% bovine serum albumin (BSA, Intergen Europe, Brussels, Belgium), 0.1% cetyl-trimethyl ammonium bromide (CTAB, Merck), 0.15% Kathone, pH 7.4.

*Sample buffer* 50 mM PBS, 0.5% CTAB, 0.05% NaN<sub>3</sub>, pH 7.4.

*Standard buffer* 50 mM PBS, 0.1% Tween 20, 5% FCS, 0.5% CTAB, 0.05% NaN<sub>3</sub>, pH 7.4.

*Substrate buffer* 0.1% p-nitrophenylphosphate (Sigma Chemicals Co., St. Louis, Missouri, USA), 0.5 M sodium carbonate, pH 9.8.

*Washing solution* 0.15 M NaCl, 0.05% Tween 20.

### Development of polyclonal antibodies against pEPO (RA-pEPO)

One rabbit was immunized with 50  $\mu$ g pEPO in 50% Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA) i.m. in a volume of 500  $\mu$ L. Four booster doses with 50  $\mu$ g pEPO in 50% Freund's incomplete adjuvant (Difco Laboratories) were given at 4, 8, 23 and 45 weeks. Sera were taken intermittently from the rabbit and analysed by ELISA against pEPO; microtitre plates (MaxiSorp, Nunc, Roskilde, Denmark) were used and the wells were filled with 100  $\mu$ L in all steps except for the postcoating step (250  $\mu$ L). After each step, the wells were washed three times. The wells were coated with the peroxidase in a concentration of 2 mg/L in coating buffer. Postcoating buffer was used to eliminate unspecific binding to the plastic. The sera were diluted 1:5 in series in reagent buffer and as a detecting antibody, SAR-IgG-ALP diluted 1:1000 in reagent buffer was used.

Substrate buffer was used for detection of bound antibodies and the absorbance was measured in a Multiskan (Labsystems, Stockholm, Sweden) at 405 nm. Serum obtained at 46 and 48 weeks (the time of sanguination) was pooled and purified on protein A Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) to obtain pure rabbit immunoglobulins [15]. The purified antibody was tested against pEPO, hEPO, pMPO and hMPO by ELISA. To estimate unspecific binding in the ELISA, a rabbit antibody against human eosinophil protein X (hEPX) was tested.

#### *In vivo experimental procedure*

Twenty-two specific pathogen-free pigs (Seropigs; Vallrum Lantbruks AB, Ransta, Sweden) of either sex were used in this study. Fourteen of them were sensitized with s.c. injections in increasing doses of 0.6–1.0 mg *A. suum* extract (Pharmacia Diagnostics AB, Uppsala, Sweden) in a suspension of Al(OH)<sub>3</sub> (3–5 mL) at the age of 3, 6 and 9 weeks, whereas the rest were left non-sensitized. The doses of *A. suum* have earlier been shown to be optimal for sensitization [16].

About 1 week after the third injection (at a body weight of 25–30 kg), the pigs were fasted overnight and premedicated with ketamine hydrochloride (Parke-Davis, Barcelona, Spain; 20 mg/kg i.m.) and anaesthesia was induced by sodium pentobarbitone (Apoteksbolaget, Umeå, Sweden; 12 mg/kg i.v.) introduced into an ear vein. Pancuronium bromide (Organon, Oss, the Netherlands; 0.2 mg/kg i.v.) was given to achieve muscle relaxation and after tracheotomy, the pigs were intubated and ventilated using a Servo ventilator (900; Siemens-Elma, Sweden). Anaesthesia was maintained by continuous i.v. infusion of pentobarbitone and pancuronium. Blood samples, drawn from a catheter put in a brachial artery, were mixed with EDTA (Merck; final concentration 10 mM). Body temperature was maintained at 38–39°C with a heating pad connected to a thermostat. 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. Airflow was measured with a heated Fleisch pneumotachygraph no. 1 connected to a Statham PM 15 E pressure transducer. The signals were sent to an AP 200 Pulmonary Computer (ConMeTech AB, Uppsala, Sweden) for on-line calculations of total lung resistance ( $R_L$ ) (see Mead [17]).  $R_L$  were continuously recorded on a Grass polygraph and simultaneously collected on an Apple Macintosh data acquisition system for analyses.

All pigs were given 28 mg *A. suum* extract, a dose causing maximal response, in 2.0 mL saline as an aerosol

generated using an ultrasonic nebulizer (NB 108, Engström Medical, Stockholm, Sweden). The aerosol was delivered over 5 min via the tracheal tube to the lower airways. Thirteen pigs (eight sensitized, five non-sensitized) were given a bolus dose of the cortisol-synthesis inhibitor metyrapone ([18]; Sigma) of 25 mg/kg followed by a continuous i.v. infusion (10 mg/kg/h).

A bronchoalveolar lavage (BAL) was performed with two aliquots of 50 mL of 37°C saline 8 h after the challenge with *A. suum* extract. The recovered volume was noted, and 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  $2.0 \times 10^5$  cells/mL and 200  $\mu$ L aliquots were prepared on glass slides using a cytocentrifuge (Cytospin 2, Shandon, Southern Products Ltd, Cheshire, UK) spinning at 500 rpm for 5 min. At the same time as the BAL was performed, a blood sample was taken. For differential blood leucocyte count, cell smears on glass slides were fixed in methanol for 5 min, stained with May-Grünwald stain (Merck) for 5 min and then with Giemsa stain (Merck) for 15 min; 200 cells were counted at a magnification of 250 $\times$ .

After BAL, the pigs were killed with an overdose of sodium pentobarbitone and lung biopsy specimens containing parenchymal tissue and bronchioles from the fifth generation (diameter 0.5–1.5 mm) were taken peripherally from the right middle lobe. Five millimetre deep skin biopsies were taken at the same time from the flank. The biopsies were snap-frozen in isopentane chilled with dry ice and were stored at –70°C until analysed.

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

#### *Immunohistochemical staining*

Blood smears made on gelatin-cromalum (Merck) treated glass slides from blood mixed with EDTA were used for immunocytochemical staining with the polyclonal antibody RA-pEPO and determination of eosinophil differential count using May-Grünwald-Giemsa staining. Also, blood leucocytes were concentrated by dextran sedimentation, and cytospin preparations were made as above and subsequently immunostained. Cytospin preparations were also made with BAL cells for immunostaining. Lung and skin biopsies were cut with a cryostat (1720 digital, Leitz, Wetzlar, Germany) to a thickness of 6  $\mu$ m, transferred to gelatin-cromalum glass slides and stored at –70°C until

immunostained. Blood smears and lung specimens were treated before staining with 50% acetone at 4°C for 30 s and thereafter with pure acetone at 4°C for 5 min. The slides were allowed to air dry and were washed in 0.05 M tris(hydroxymethyl)aminomethane-HCl in saline, pH 7.6 (TBS) for at least 5 min at room temperature (25°C) and the washing was repeated after antibody incubations. All antibodies were diluted in 4% BSA in TBS and incubations were performed in humid chambers at room temperature. The specimens were first incubated with 10% normal sheep serum for 10 min to reduce unspecific binding and then for 30 min with RA-pEPO diluted 800 times. SAR-IgG-ALP diluted 25 times was incubated for 30 min and detection of bound antibodies was made by Dako Fast Red Substrate System (Dako Corporation, Carpinteria, California, USA) containing naphtholphosphate, Fast Red and levamisole, incubated for 20 min. The glass slides were then washed three times in TBS followed by distilled water. The specimens were counterstained with Mayer's haematoxylin for 30 s. After flushing with tap water for 10 min, the specimens were mounted with Kaiser's glycerol gelatin (Merck). As controls, the primary antibody was replaced for every specimen with normal rabbit immunoglobulins purified by means of protein A. No staining was observed in these tests. For comparison between May-Grünwald-Giemsa and RA-pEPO-staining blood smears were taken before and 9 h after metyrapone treatment and evaluated in light microscopy at a magnification of 250× and the ratios of eosinophil vs mononuclear cell numbers were calculated.

Two sections per lung biopsy specimen, stained with RA-pEPO were coded and analysed at magnification 100× in light microscopy and divided into four classes; class 0 corresponding to < 5, class 1 to 5-50, class 2 to 50-200 and class 3 to > 200 eosinophils per mm<sup>2</sup>. Skin sections (three per biopsy specimen) stained with RA-pEPO were evaluated at magnification 100× in light microscopy.

#### *Monoclonal antibodies raised against hEPO and pEPO*

Positive mouse hybridomas obtained after immunization with hEPO (Pharmacia AB, Diagnostics) were tested against pEPO by ELISA and hybridomas reacting with both hEPO and pEPO were cloned. The isotypes were determined by ELISA with subclass-specific antibodies from Bionetics Laboratory Products (Litton Bionetics Inc., Kensington, Maryland, USA). The monoclonal antibody showing the highest affinity in a prototype sandwich-ELISA was cultivated in serum-free medium (Pharmacia AB, Diagnostics) and purified by means of hydrophobic interaction using methods described by

Hrkál and Rejnková [19], followed by gel-filtration on Superdex 200 prep grade (Pharmacia Biotech AB). The monoclonal antibody was then conjugated with ALP at a ratio of 3:1 using *N*-succinimidyl 3-(2-pyridyldithio) propionate as a linker [20]. The conjugated antibody was fractionated on Superose 6 (Pharmacia Biotech AB). This conjugate (MA-hEPO-ALP) was used as the detecting antibody in the sandwich-ELISA.

#### *Detection of pEPO and BAL fluid*

pEPO was measured using a sandwich-ELISA; the wells were filled with 100 µL in all steps except for postcoating (250 µL) and samples and standards (200 µL). After each step, washing solution was applied three times. Wells were coated with RA-pEPO (1 mg/L) in coating buffer overnight at 8°C, and then incubated with postcoating buffer for at least 1 day at 8°C. The plates could be stored in this buffer for at least 2 weeks. The standard curve was made by dilution of purified pEPO in standard buffer. The BAL samples were mixed with sample buffer (10 µL–200 µL). The samples and standards in triplicate were incubated overnight at 8°C during slow shake. MA-hEPO-ALP was diluted 1:750 in reagent buffer and incubated 1 h at 8°C during vigorous shake and substrate buffer was then incubated 1 h at 37°C, and the absorbance was measured in a Labsystems Multiskan at 405 nm.

#### *Total protein determination*

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<sup>2+</sup> produced in the reaction of protein with alkaline Cu<sup>2+</sup>. The range of the test was 10–1200 µg/mL.

#### *Determination of plasma cortisol*

Plasma was obtained by centrifugation of EDTA blood at 4°C, 680 × g for 10 min and stored at –70°C until analysis of cortisol using a radio-immunoassay (Orion Diagnostica AB, Trosa, Sweden), with a detection limit of 3–5 nM.

#### *Statistics*

Values are presented as mean ± SEM. The detection limit of the sandwich-ELISA was defined as the lowest peroxidase concentration which gave a response 2 sds above the buffer control as determined using 10 replicates. The values of the samples were calculated

**Table 1.** Maximal acute and late (within 8 h) increase in total lung resistance ( $R_L$ ). Values are presented as mean (%)  $\pm$  SEM

|             | Sensitized high cortisol<br><i>n</i> = 6 | Sensitized low cortisol<br><i>n</i> = 8 | Non-sensitized high cortisol<br><i>n</i> = 3 | Non-sensitized low cortisol<br><i>n</i> = 5 |
|-------------|------------------------------------------|-----------------------------------------|----------------------------------------------|---------------------------------------------|
| $R_L$ acute | 96 $\pm$ 27                              | 67 $\pm$ 11                             | 0 $\pm$ 5                                    | 0 $\pm$ 2                                   |
| $R_L$ late  | 14 $\pm$ 19                              | 57 $\pm$ 8                              | 1 $\pm$ 7                                    | 7 $\pm$ 6                                   |

from the standard curves fitted by cubic spline and corrected for the dilution of the sample with sample buffer.

Mann-Whitney *U*-test was used for comparison between the two groups of BAL samples, and values below the detection limit were set to the value of the detection limit. For correlation analysis, Spearman rank correlation test was used.

## Results

### *In vivo experiments*

The plasma cortisol levels were in the metyrapone-treated pigs  $36.7 \pm 2.6$  nM (*n* = 13) and in the non-metyrapone-treated pigs  $399 \pm 41$  nM (*n* = 9). The higher value was probably due to the stress of anaesthesia and surgery. Since the normal morning cortisol levels in the pig are ranging from 75–140 nM [21] the groups are from now on called low- and high-cortisol pigs, respectively.

In all sensitized pigs an acute increase in  $R_L$  was noted with a maximum at about 10 min and which resolved within 1 h. A late increase in  $R_L$  was seen, starting at about 4 h after allergen challenge. The bronchial obstruction, as measured as maximal acute and late (last time point 8 h) increase in  $R_L$  are presented in Table 1.

### *Characterization of polyclonal antibodies against pEPO*

RA-pEPO was tested in an ELISA using pEPO, hEPO, pMPO and hMPO coated onto the wells of a microtiter plate. The results showed that the antibody had high affinity to pEPO; crossreactivity against hEPO was 50%, against hMPO, 5% and against pMPO, less than 0.2%. An absorbance of 0.14 was seen at the highest concentration of the rabbit antibody with irrelevant specificity (anti-hEPX), suggesting the presence of unspecific binding at higher concentrations of rabbit antibodies to the cationic protein. In microtitre plates not coated with peroxidase, no binding at all of the rabbit antibodies was seen.

Comparison of blood smears assessed using May-Grünwald-Giemsa staining and those assessed with RA-pEPO/Mayer's haematoxylin suggested that only eosinophils were stained by RA-pEPO (Fig. 1). This was also confirmed on cytospin preparations of BAL cells, where RA-pEPO likewise did not stain macrophages (not shown). In the blood smears, 35–65% of the leucocytes were neutrophils, and 0–1% were basophils. Fig. 2a shows eosinophils in blood leucocyte cytospin preparations stained with RA-pEPO. Using the same antibody, cells in lung tissue specimens could also be stained as shown in Fig. 2b. Figure 3 shows lung biopsy specimens taken 8 h after allergen challenge from a high-cortisol, non-sensitized pig (Fig. 3a), and a low-cortisol, sensitized pig (Fig. 3b). Scoring of the eosinophil density in the lung parenchyma is shown in Fig. 4a. Very few eosinophils were found in the high-cortisol pigs. In low-cortisol, non-sensitized pigs, slightly increased numbers of eosinophils could be detected in the lung parenchyma.

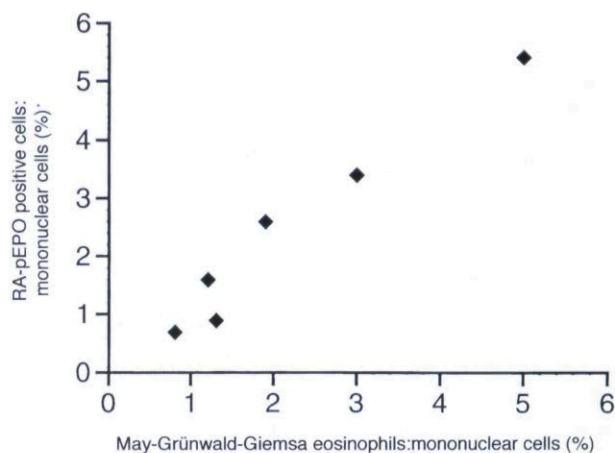


**Fig. 1.** Duplicates of acetone-treated blood smears from three pigs before and 9 h after metyrapone treatment, were stained with May-Grünwald-Giemsa staining or with RA-pEPO. The diagram shows the ratio (%) between eosinophils and mononuclear cells as judged by the two staining techniques. Correlation analysis was performed using Spearman rank correlation test.  $r = 0.94$ .  $P < 0.05$ .

The highest number of eosinophils was found in the sensitized, low-cortisol pigs and, in these animals, also near the epithelium of the bronchioles (Fig. 3b). In the skin, very few eosinophils were found and there were no significant differences between the groups.

#### *Characterization of monoclonal antibodies against hEPO*

Of 17 different hybridomas positive to hEPO, three reacted with hEPO and pEPO to approximately the

same extent. Antibodies from seven other hybridomas reacted to a lesser extent with pEPO, whereas the rest were negative to pEPO. The three hybridomas with the highest crossreactivity with pEPO were cloned and showed to be of IgM, IgA and IgG<sub>1</sub> isotypes. When tested in a sandwich-ELISA with pEPO in solution, the IgA monoclonal antibody showed the highest affinity for pEPO. This monoclonal antibody was purified and conjugated with ALP.

#### *Immunoassay for detection of pEPO in BAL*

The detection limit of the assay was determined to be

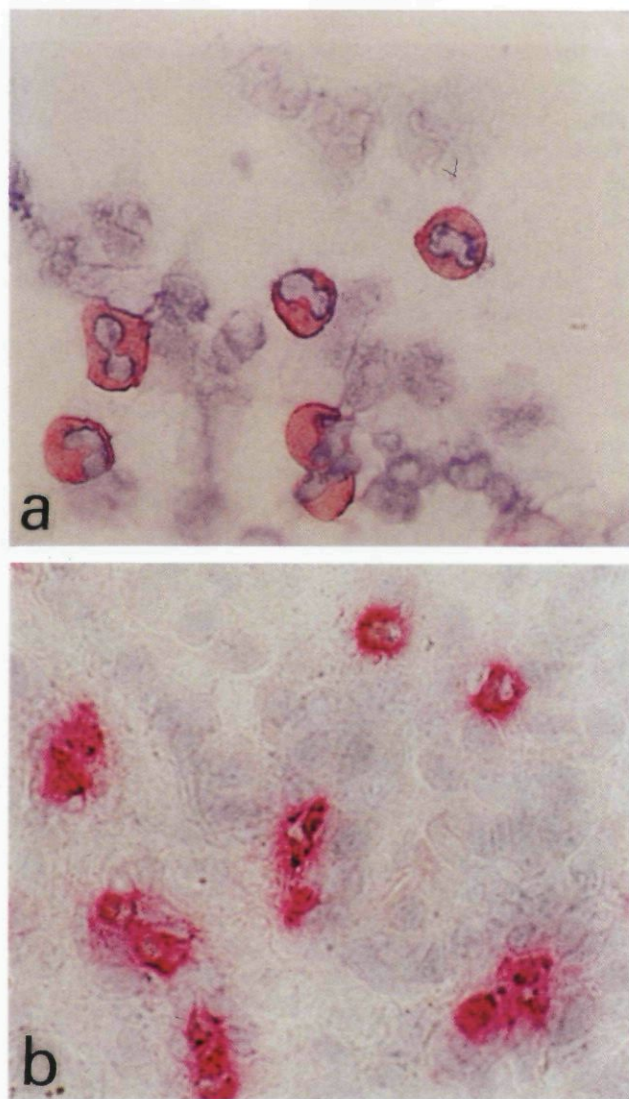


**Fig. 2.** Detection of RA-pEPO-positive cells in an acetone-treated cytopspin preparation of blood leucocytes (a) and a lung tissue section (b) from a low-cortisol, sensitized pig. The original magnification was 400 $\times$  and counterstaining was done with Mayer's haematoxylin. Note the bilobular nucleus, characteristic of the eosinophil granulocyte, in the RA-pEPO-positive, red cells.

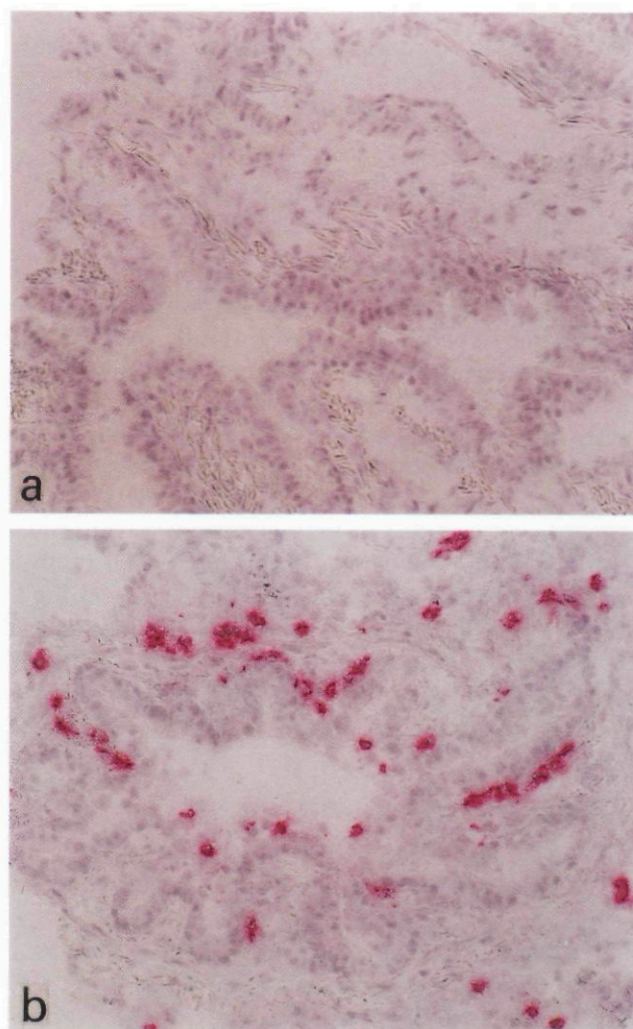


**Fig. 3.** Acetone-treated sections from frozen lung tissue including a bronchiole stained with RA-pEPO-antibody. (a) shows a high-cortisol, non-sensitized pig scored as class 0 and (b) shows a low-cortisol, sensitized pig scored as class 3. The original magnification was 200 $\times$  and counterstaining was done with Mayer's haematoxylin.

0.15  $\mu\text{g/L}$  and no crossreactivity was found for pMPO or hMPO within the range of 0.15–40  $\mu\text{g/L}$  (Fig. 5). The crossreactivity to hEPO was found to be approximately 5% in this sandwich ELISA. One BAL sample (concentration 1.49  $\mu\text{g/L}$ ) serially diluted two, four and eight times in sample buffer paralleled the standard curve well; observed/expected values were within the range of 82–110%.

#### Measurements of BAL samples

The concentration of free EPO in BAL fluid samples was

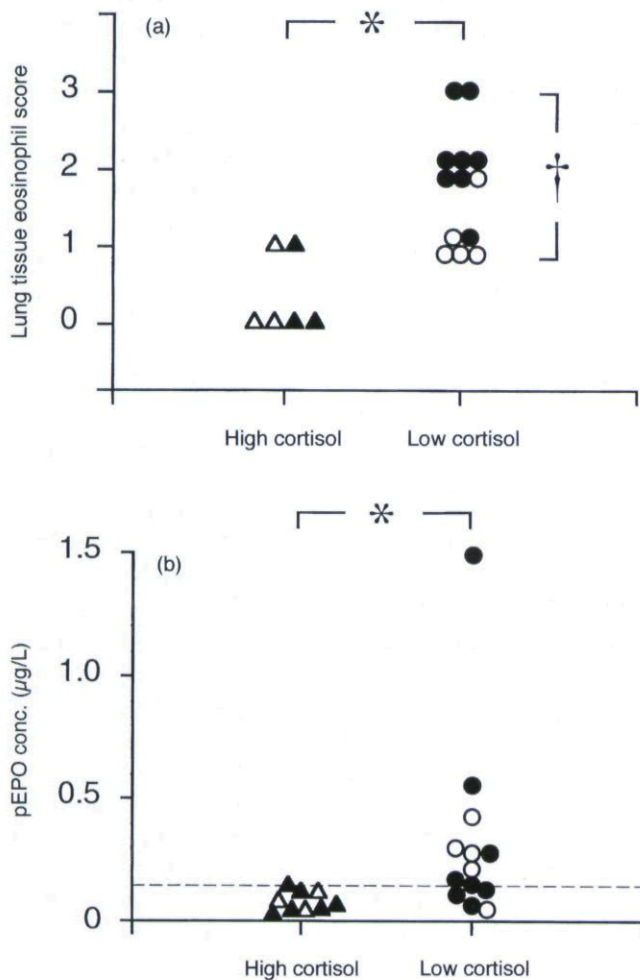


**Fig. 4.** (a) Scoring of lung tissue eosinophils (RA-pEPO-positive cells) and (b) concentration of free pEPO in BAL fluid in sensitized, high-cortisol pigs ( $\blacktriangle$ ), non-sensitized, high-cortisol pigs ( $\triangle$ ), sensitized, low-cortisol pigs ( $\bullet$ ) and non-sensitized, low-cortisol pigs ( $\circ$ ). \* indicates  $P < 0.05$  when comparing high- and low-cortisol pigs, Mann-Whitney  $U$ -test. † indicates  $P < 0.05$  when comparing sensitized, metyrapone-treated and non-sensitized, metyrapone-treated pigs, Mann-Whitney  $U$ -test. Broken line represents the detection limit.

determined using the sandwich-ELISA. No pEPO was detected in the high-cortisol pigs (Fig. 4b). Significantly elevated pEPO levels were noted in low-cortisol pigs compared to high-cortisol animals. However, there was no significant difference between pEPO levels in the sensitized and non-sensitized pigs pretreated with metyrapone, although one sensitized animal showed a marked elevation of pEPO concentration in BAL fluid.

#### Correlation analysis

All pigs were included in the correlation analysis. The number of blood eosinophils per mL at 8 h after allergen challenge correlated significantly with the lung tissue eosinophil score (Fig. 6). However, no correlation was found between either of blood eosinophil number at 8 h, lung tissue eosinophil score and the concentration of eosinophils on the other hand, and pEPO in BAL fluid on the other. Also, skin eosinophil evaluation did not correlate with blood eosinophil number or lung tissue eosinophil score. The concentration of pEPO and total protein in BAL fluid did not correlate ( $r = 0.23$ ,  $P = 0.48$ ), and when the pEPO concentration was divided by the total protein content the difference between high- and low-cortisol pigs seen in Fig. 4b was not changed.

#### Discussion

The eosinophil granulocyte possesses antihelminthic activity and is probably involved in host defence against parasites [11,21]. The eosinophil is also associated with

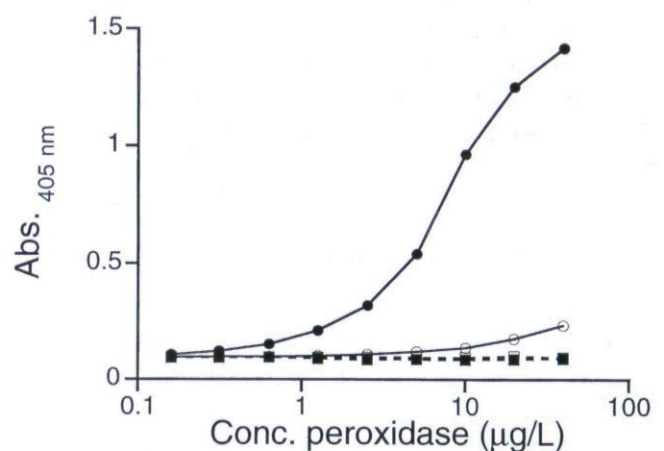


**Fig. 5.** The peroxidases pEPO ( $\bullet$ ), hEPO ( $\circ$ ), pMPO ( $\blacksquare$ ) and hMPO ( $\square$ ) were tested in the sandwich-ELISA at a concentration of 0.15–40  $\mu\text{g/L}$ . No crossreactivity was found for pMPO or hMPO, while approximately 5% crossreactivity was found for hEPO.



assays using *o*-phenylenediamine (OPD) as hydrogen donor [7]. However, this method is not sensitive enough to detect free prereleased EPO in BAL fluid, and only cell suspensions or homogenates can be tested. Furthermore, to avoid the confounding influence of the activity of MPO, the contamination with neutrophils must be low. A modified and more sensitive method based on OPD has recently been developed by White *et al.* [27]. In this assay, which has a detection limit of 30 µg/L, released hEPO from purified and enriched human eosinophils could be detected. Homovanillic acid has been suggested to be oxidated specifically by EPO and this specificity has been employed to distinguish between EPO and MPO activity [28], but the sensitivity of both of these activity assays would probably be too low to detect free pEPO in BAL fluid in the pig, since immunoassay showed these levels to be below 1.5 µg/L. In the presently described sandwich-ELISA with RA-pEPO as catching antibody and MA-hEPO-ALP as detecting antibody, the detection limit was estimated to be 0.15 µg/L and there was no crossreactivity with pMPO in the range of 0.15–40 µg/L. The low detection limit was achieved by letting the equilibration between RA-pEPO and pEPO take place at 8°C for 16 h. Furthermore, a cationic detergent (CTAB) was added to the BAL-samples to prevent unspecific cationic binding.

In spite of the sensitivity of the test, pEPO levels in most of the samples from the high-cortisol pigs were below the detection limit. However, in the low-cortisol pigs nine out of 13 samples showed pEPO levels above 0.15 µg/L, suggesting that EPO-release is controlled by endogenous cortisol. In the present study we saw a striking correlation between the numbers of blood eosinophils and of cells containing pEPO in the lung (tissue eosinophils). This, together with the fact that eosinophil numbers in the skin did not at all correlate with blood eosinophils and did not change with treatment, suggests that in this model with local allergen provocation in the lower airways the newly recruited eosinophils are destined specifically to enter lung tissue. Also, the presence of eosinophils close to the epithelium in sensitized, low-cortisol animals indicates chemotaxis towards the bronchial lumen induced by allergen challenge. No clear-cut correlation was found between lung tissue and BAL eosinophil numbers or pEPO levels in BAL fluid, suggesting that 8 h is too short a time for the eosinophil to make its way from the circulation to the bronchial epithelium. This may possibly be overcome by using a more allergenic material in the sensitization procedure to enable stronger acute reactions than those elicited in the present animals. However, since this study was aiming at finding correlations between eosinophil recruitment and late airways

obstruction, and the latter is profound at 8 h, this time point was chosen for evaluation. Due to the fact that BAL fluid EPO levels in some of the sensitized, low-cortisol pigs were below the detection limit, no correlation between this marker of eosinophil activation and the degree of late-phase airways obstruction could be seen. However, the difference in BAL fluid EPO levels between high- and low-cortisol pigs seem to reflect lung tissue eosinophil activity and not the degree of vascular leakage, since EPO levels did not follow total protein content in BAL fluid. Higher numbers of blood eosinophil [4] and lung tissue eosinophils (present data) were noted in non-sensitized, low-cortisol pigs compared to non-metyrapone-treated animals. This may merely reflect cortisol-induced eosinophil suppression in non-metyrapone animals, and that metyrapone treatment restores normal blood and tissue eosinophil count.

In conclusion, using the herein described newly developed methods we have shown a profound infiltration of eosinophils into lung tissue after allergen challenge in metyrapone-treated, sensitized pigs. In contrast, in non-metyrapone treated animals with high plasma cortisol levels, no eosinophil recruitment could be seen. We also describe for the first time direct measurements of EPO in BAL fluid, which in the pig was under marked cortisol control.

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