British Journal of Pharmacology (1996) 119, 1484–1490

Effect of dexamethasone and endogenous corticosterone on airway hyperresponsiveness and eosinophilia in the mouse

[1]J.J. De Bie, E.M. Hessel, I. Van Ark, B. Van Esch, G. Hofman, F.P. Nijkamp & A.J.M. Van Oosterhout

Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

1 Mice were sensitized by 7 intraperitoneal injections of ovalbumin without adjuvant (10 μg in 0.5 ml of sterile saline) on alternate days and after 3 weeks exposed to either ovalbumin (2 mg ml^{-1} in sterile saline) or saline aerosol for 5 min on 8 consecutive days. One day before the first challenge, animals were injected intraperitoneally on a daily basis with vehicle (0.25 ml sterile saline), dexamethasone (0.5 mg kg^{-1}) or metyrapone (30 mg kg^{-1}).

2 In vehicle-treated ovalbumin-sensitized animals ovalbumin challenge induced a significant increase of airway responsiveness to metacholine both *in vitro* (27%, $P<0.05$) and *in vivo* (40%, $P<0.05$) compared to saline-challenged mice. Virtually no eosinophils could be detected after saline challenge, whereas the numbers of eosinophils were significantly increased ($P<0.01$) at both 3 and 24 h after the last ovalbumin challenge ($5.48\pm3.8\times10^3$ and $9.13\pm1.7\times10^3$ cells, respectively). Furthermore, a significant increase in ovalbumin-specific immunoglobulin E level (583 ± 103 units ml^{-1}, $P<0.05$) was observed after ovalbumin challenge compared to saline challenge (201 ± 38 units ml^{-1}).

3 Plasma corticosterone level was significantly reduced (−92%, $P<0.001$) after treatment with metyrapone. Treatment with metyrapone significantly increased eosinophil infiltration ($17.4\pm9.93\times10^3$ and $18.7\pm2.57\times10^3$ cells, $P<0.05$ at 3 h and 24 h, respectively) and potentiated airway hyperresponsiveness to methacholine compared to vehicle-treated ovalbumin-challenged animals. Dexamethasone inhibited both *in vitro* and *in vivo* hyperresponsiveness as well as antigen-induced infiltration of eosinophils (0, $P<0.05$ and $0.7\pm0.33\times10^3$ cells, $P<0.05$ at 3 h and 24 h, respectively). Metyrapone as well as dexamethasone did not affect the increase in ovalbumin-specific immunoglobulin E levels after ovalbumin challenge (565 ± 70 units/ml^{-1}; $P<0.05$; 552 ± 48 units ml^{-1}, $P<0.05$ respectively).

4 From these data it can be concluded that exogenously applied corticosteroids can inhibit eosinophil infiltration as well as airway hyperresponsiveness. *Vise versa*, endogenously produced corticosteroids play a down-regulating role on the induction of both eosinophil infiltration and airway hyperresponsiveness.

Keywords: Airway hyperresponsiveness; eosinophilia; ovalbumin; immunoglobulin E; corticosteroids

Introduction

Asthma is characterized by reversible airway obstruction and an increase in airway hyperresponsiveness to various bronchoconstrictive stimuli (Nadel & Sheppard, 1985). It is generally accepted that airway inflammation plays an important role in the increased airway responsiveness found in this disease (Djukanovic *et al.*, 1990). This is based on the association between airway inflammation, especially infiltration of eosinophils and mononuclear cells in both severe (Dunnil, 1978) and in mild asthmatics (Beasly *et al.*, 1989), and an increase in nonspecific airway responsiveness or late asthmatic responses (O'Byrne *et al.*, 1987; Cartier *et al.*, 1982). Studies in different animal models have also revealed an evident relationship between antigen-induced infiltration of inflammatory cells, mostly eosinophils (Richards *et al.*, 1990), and late asthmatic responses (Iijima *et al.*, 1987) or airway hyperresponsiveness (Elwood *et al.*, 1992a).

Corticosteroids are one of the few successful therapeutic agents in asthma. This has been demonstrated in several clinical studies in which treatment of asthmatics with corticosteroids decreased both airway hyperresponsiveness and inflammatory cell infiltration (see Nijkamp & Van Oosterhout, 1993). Endogenous corticosteroids are also thought to be important in asthma. In man, cortisol is secreted in a diurnal pattern (Weitzman *et al.*, 1971) which may be involved in nocturnal exacerbations (Kallenbach *et al.*, 1988). During these nightly exacerbations (Clark & Hetzel, 1977) an increase in inflammatory cell numbers in lung tissue occurs (Mackay *et al.*, 1994) whereas endogenous corticosteroid levels are decreased. Interestingly, it has been shown that factors released from activated leukocytes can increase the level of endogenous corticosteroids (Besedovsky *et al.*, 1981). This suggests that endogenous corticosteroids may act as a negative feed-back mechanism during activation of immune and inflammatory responses. However, the precise role of endogenous corticosteroids in developement of asthma-related symptoms remains to be elucidated.

Recently, we have described a model for allergic asthma (Hessel *et al.*, 1994; 1995; 1996) in which both airway hyperresponsiveness and infiltration of inflammatory cells occur after ovalbumin challenge in sensitized mice. In the present study, we were interested in the effects of exogenously administered corticosteroids and the role of endogenously produced corticosteroids on the development of asthma symptoms in this model, i.e. airway hyperresponsiveness, antigen-specific immunoglobulin E production and inflammatory cell infiltration. Therefore, animals were treated with the synthetic steroid dexamethasone or with metyrapone, which inhibits endogenous corticosteroid production.

Methods

Sensitization and challenge

Specified pathogen free male BALB/c mice (6–8 weeks) were obtained from the breeding colony of the National Institute for

[1]Author for correspondence at: Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O.Box 80.0082, 3508 TB Utrecht, The Netherlands.

Public Health and the Environment, Bilthoven, The Netherlands. The mice were housed in macrolon cages and provided with food and water *ad libitum*. Active sensitization was performed by 7 intraperitoneal injections of 10 μg ovalbumin (grade II) in 0.5 ml pyrogen free saline on alternate days. This sensitization procedure has been shown to induce high titres of total immunoglobulin E antibodies in the serum, of which 80% was ovalbumin-specific immunoglobulin E (Hessel *et al.*, 1995). Four weeks after the last injection, the mice were exposed either to 8 ovalbumin (2 mg ml^{-1} in sterile saline) or 8 saline aerosols, on consecutive days (1 aerosol per day). Mice were exposed for 5 min with a maximal group of 6 animals. The aerosol was generated with an ultrasonic nebulizer (Medix 8001, particle size 3–5 μm) connected to a plexiglas exposure chamber with a volume of 5 l. One day before the challenge started, the mice were injected intraperitoneally on a daily base with either 0.25 ml dexamethasone (0.5 mg kg^{-1} in sterile saline) or 0.25 ml metyrapone (30 mg kg^{-1} in sterile saline). Control mice were injected with 0.25 ml sterile saline.

Airway responsiveness in vitro

In order to measure airway responsiveness *in vitro*, tracheae were isolated and the responsiveness to methacholine was measured in an organ bath as originally described by Garssen *et al.* (1990). In previous experiments it was established that the optimal time-point to measure tracheal hyperresponsiveness to methacholine *in vitro* was at 3 h after the last challenge (Hessel *et al.*, 1994). At this time-point the mice were anaesthetized by intraperitoneal injection of 0.25 ml sodium pentobarbitone (60 mg ml^{-1}). Abdomen and chest were opened and the abdominal aorta was incised. Between the 10th and 11th tracheal ring below the larynx a small incision was made, and a flexible polyethylene cannula (PE 50, Intramedic, Clay Adams, NJ, U.S.A.) was inserted into the trachea and fixed with a ligature for lavage. During the preparation procedure the trachea was humidified with Krebs bicarbonate solution (composition mM: NaCl 118.1, $NaHCO_3$ 25.0, glucose 11.1, $NaH_2PO_2.H_2O$ 1.0, KCl 4.7, $MgCl_2.6H_2O$ 0.5, $CaCl_2.6H_2O$ 2.5, pH 7.4). Nine tracheal rings above the cannula were isolated, and transferred to a petri dish containing Krebs solution. Remaining connective tissue was removed from the trachea by use of a binocular microscope and thereafter the trachea was mounted in an organ bath (15 ml) filled with Krebs solution. The organ bath was constantly aerated with a mixture of 95% O_2 and 5% CO_2 and a temperature of 37°C was maintained. Changes in trachael muscle contraction were measured with an isometric transducer (Harvard Bioscience, Boston, MA, U.S.A.), connected to a 2-channel pen recorder (Servoger, type SE-220). After a stabilization period (45 min) at basal tension (1 g), cumulative concentration-response curves were made with methacholine. The concentration-response curves started with 1×10^{-8} M and ended with a concentration of 3×10^{-4} M methacholine at which maximal contraction was reached. Concentrations of methacholine were prepared in saline and kept on ice for the duration of the experiment. Results are expressed in mg contraction.

Airway responsiveness in vivo

Airway responsiveness was measured *in vivo* at 24 h after the last aerosol exposure, by an air-overflow pressure method (Raeburn *et al.*, 1992). In previous experiments it was established that this time-point was optimal for measuring airway hyperresponsiveness *in vivo* (Hessel *et al.*, 1996). With this method the bronchial resistance to inflation is measured. The mice were anaesthetized by intraperitoneal injection of urethane (2 g kg^{-1}), and placed on a heated blanket (30°C). The trachea was cannulated and a small polyethylene catheter (PE 10, Intramedic, Clay Adams, NJ, U.S.A.) was placed in the jugular vein for intravenous administration. The spontaneous breathing was suppressed by intravenous injection of tubocurarine chloride (3.3 mg kg^{-1}). When it stopped, the tracheal cannula was attached to a respiration pump (C.F. Palmer, London, U.K.). The inflation volume of the pump was 0.8 ml per beat of which the animal inhales approximately 0.1 ml with 190 strokes min^{-1}. During inflation P_{O_2} and P_{CO_2} levels stayed within physiological range. A pressure transducer (MPB-6207, Depex, Bilthoven, The Netherlands) was located between the trachea and the respiration pump in order to measure changes in the bronchial resistance to inflation. Pressure signal was recorded breath-by-breath on a Graphtex thermal arraycorder (Ankersmit, Breda, The Netherlands). At time intervals of at least 4 min and after the response was returned to baseline level, doubling doses of methacholine ranging from 40 μg kg^{-1} to 1280 μg kg^{-1} were administered. To determine whether the observed hyperresponsiveness was not specific for methacholine alone, additional experiments were performed in which ovalbumin-sensitized animals were exposed to either saline or ovalbumin aerosols and dose-response curves were obtained with methacholine (ranging from 40 μg kg^{-1} to 1280 μg kg^{-1}) or 5-hydroxytryptamine (5-HT) (ranging from 20 μg kg^{-1} to 640 μg kg^{-1}). At the end of the dose-response curve, the maximal response was determined by clamping the tracheal cannula. The increase in air-overflow pressure was measured at its peak and expressed as % increase of the maximal response. At least 6 mice were evaluated per group.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed in the same animals that were used for airway hyperresponsiveness measurements. In pilot experiments it was found that combining these techniques had no effect on the total number of cells derived from the lavage nor on the appearance of the different cell types. Mice were lavaged 5 times through the tracheal cannula with 1 ml aliquots of pyrogen free saline at 37°C. The BAL was kept on ice until further processing. The BAL cells were washed with cold phosphate-buffered saline (PBS) (400 *g*, 4°C, 5 min) and the pellet was resuspended in 200 μl cold PBS. A Bürker-Türk chamber was used to count the total number of BAL cells. For differential BAL cell counts cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Düdingen, Switzerland). After coding, all cytospin preparations were evaluated by one observer by oil immersion microscopy. Cells were identified and differentiated into mononuclear cells, neutrophils and eosinophils by standard morphology. At least 400 cells per cytospin preparation were counted and the absolute number of each cell type was calculated.

Determination of ovalbumin-specific immunoglobulin E serum levels

Ninety-six well microplates (Nunc A/S, Roskilde, Denmark) were coated with 2 μg ml^{-1} chimeric fusion protein of the human high affinity immunoglobulin E receptor and human immunoglobulin G (FcεR1-IgG) diluted in PBS. After 12–24 h incubation at 4°C the plates were washed 5 times with PBS supplemented with 0.05% Tween-20 (PBT). Thereafter, the plates were blocked with ELISA buffer (2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.5% BSA, 0.05% Tween-20, pH 7.2) and left to incubate at room temperature for 1 h on an orbital shaker. Afterwards, serum samples were added to the plates and left to incubate on an orbital shaker at room temperature for 2 h. OVA-IgE reference standard dilution series were treated accordingly. The standard was obtained by intraperitoneal immunization of mice with ovalbumin, and arbitrarily assigned a value of 10,000 units ml^{-1} OVA-specific immunoglobulin E. After washing, 10 μg ml^{-1} of chicken egg albumin in ELISA buffer was added to each well and after incubation at room temperature for 1 h washing procedures were repeated. Horse-radish peroxidase-conjugated goat anti-OVA antibody was diluted in ELISA buffer and added to each well. Incubation was continued for 1 h followed by washing

procedures. The last step was a 15–30 min incubation at room temperature with 10 mM OPD substrate solution after which the reaction was stopped by adding H_2SO_4 (4 M). OD was read at λ 492 nm by use of a Titertek Multiskan (Flow Labs., Irvine, U.K.).

Determination of plasma corticosterone levels

Plasma corticosterone levels were measured in blood samples collected at 3 h after the last challenge with a commercially available radioimmunoassay kit (ICN Biochemicals Inc., Costa Mesa, CA, U.S.A.) and plasma samples of vehicle or metyrapone-injected animals were treated according to the manufacturer's instructions. This ^{125}I radioimmunoassay kit for rats and mice corticosterone is highly specific with a cross-reactivity to desoxycorticosterone of 0.34%.

Drugs and chemicals

Ovalbumin (chicken egg albumin crude grade II), *o*-phenylenediamine, 3-amino-1,2,4-triazole, dexamethasone, metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) and 5-HT were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) recombinant human FcεR1-IgG, horse-radish peroxidase-conjugated goat anti-OVA antibody and OVA-IgE reference standard were generously provided by Dr P.M. Jardieu, Genentech Inc. (South San Francisco, CA, U.S.A.). Urethane and methacholine (acetyl-β-methylcholine) were purchased from Janssen Chimica (Beerse, Belgium), tubocurarine chloride from Nogepha (Netherlands), sodium pentobarbitone (Nembutal) from Abott Laboratories (North Chicago, IL, U.S.A.) and Tween-20 from Merck (Darmstadt, Germany).

Statistical analysis

Unless stated otherwise, data are expressed as arithmetic average ± s.e.mean and comparisons between 2 groups were made by Student's *t* test (unpaired, two-tailed). A difference was considered to be significant when $P<0.05$. Concentration-response curves were tested with an analysis of variance (ANOVA, two-way). Total BAL cell number and the numbers of the various BAL cell types were tested with an analysis of variance (ANOVA). For cell types with a very low number in control animals (i.e. neutrophils and eosinophils) a Poisson distribution was assumed. Statistical analyses were carried out with SPSS/PC$^+$, version 4.0.1 (SPSS Inc., Chicago, IL, U.S.A.) or GLIM, version 4.0 (NaG Inc., Oxford, U.K.).

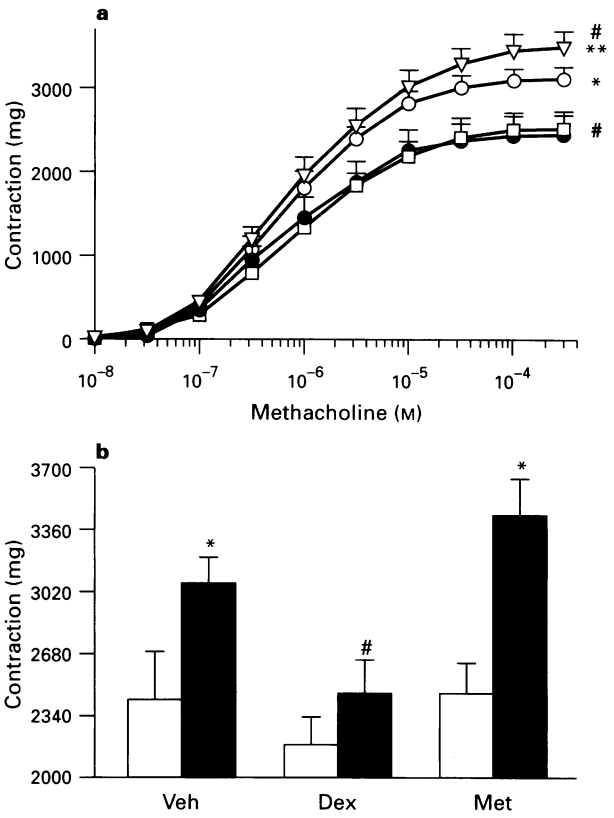

Figure 1 (a) Tracheal responsiveness to methacholine measured in ovalbumin-sensitized mice treated with vehicle (●, ○) and challenged with either saline (●) or ovalbumin (○) or treated with metyrapone (△) or dexamethasone (□) and challenged with ovalbumin at 3 h after the last challenge. No differences were observed between saline-challenged animals of all different treatment groups (data not shown). Results are expressed as arithmetic average ± s.e.mean ($n=6$ per group): *Significantly different from saline-challenged animals ($P<0.05$, ANOVA); **$P<0.01$, ANOVA; # significantly different from vehicle-treated ovalbumin-challenged animals ($P<0.05$, ANOVA). (b) Differences in maximal response at the highest dose of methacholine after saline (open columns) or ovalbumin challenge (solid columns) of animals treated with either vehicle (Veh), dexamethasone (Dex) or metyrapone (Met). Results are expressed as arithmetic average ± s.e.mean ($n=6$ per group). *Significantly different from saline-challenged animals ($P<0.05$, Student's *t* test); #significantly different from vehicle-treated ovalbumin-challenged animals ($P<0.05$, Student's *t* test).

Results

Airway responsiveness in vitro

At 3 h after the last challenge, responsiveness to methacholine in vehicle-treated ovalbumin-sensitized mice was significantly increased compared to saline-challenged control animals ($P<0.05$) (Figure 1a). The maximal increase in responsiveness which was observed at the highest dose of methacholine (3×10^{-4} M) was 27% ($P<0.05$) (Figure 1b). In metyrapone-treated mice responsiveness after ovalbumin challenge was significantly increased ($P<0.01$) compared to saline-challenged mice (Figure 1a). The concentration-response curve of ovalbumin-challenged mice after metyrapone treatment was also significantly increased compared to vehicle-treated ovalbumin-challenged animals ($P<0.05$). The increase at the highest dose of methacholine was 40% compared to metyrapone-treated saline-challenged animals (Figure 1b). In contrast, in dexamethasone-treated animals, hyperresponsiveness in ovalbumin-challenged mice was not significantly different from their saline-challenged controls (Figure 1a). The maximal response to methacholine after dexamethasone treatment was significantly ($P<0.05$) decreased with 20% ($P<0.05$) compared to vehicle-treated ovalbumin-challenged animals (Figure 1b). No significant differences in responsiveness were observed among saline-challenged animals of the different treatment groups (data not shown). Furthermore, no differences were observed in EC_{50} values of the methacholine concentration-response curves between saline or ovalbumin-challenged animals in all different treatment groups (data not shown).

Airway responsiveness in vivo

In vehicle-treated animals ovalbumin-challenged animals a significant increase in responsiveness was observed at 24 h after the last challenge at doses of methacholine ranging from 320 μg kg^{-1} to 1280 μg kg^{-1} compared to their saline-challenged controls (Figure 2a). The increase was 48% at 320 μg kg^{-1}, 58% at 640 μg kg^{-1} and 40% at 1280 μg kg^{-1}. In metyrapone-treated ovalbumin-challenged animals a significant increase in responsiveness to methacholine was observed at doses ranging from 160 μg kg^{-1} to 1280 μg kg^{-1} (Figure 2b). This observed increase ranged from 98% at 160 μg kg^{-1}, 61% at 320 μg kg^{-1}, 50% at 640 μg kg^{-1} to 30% at 1280 μg kg^{-1}. After metyrapone treatment a sig-

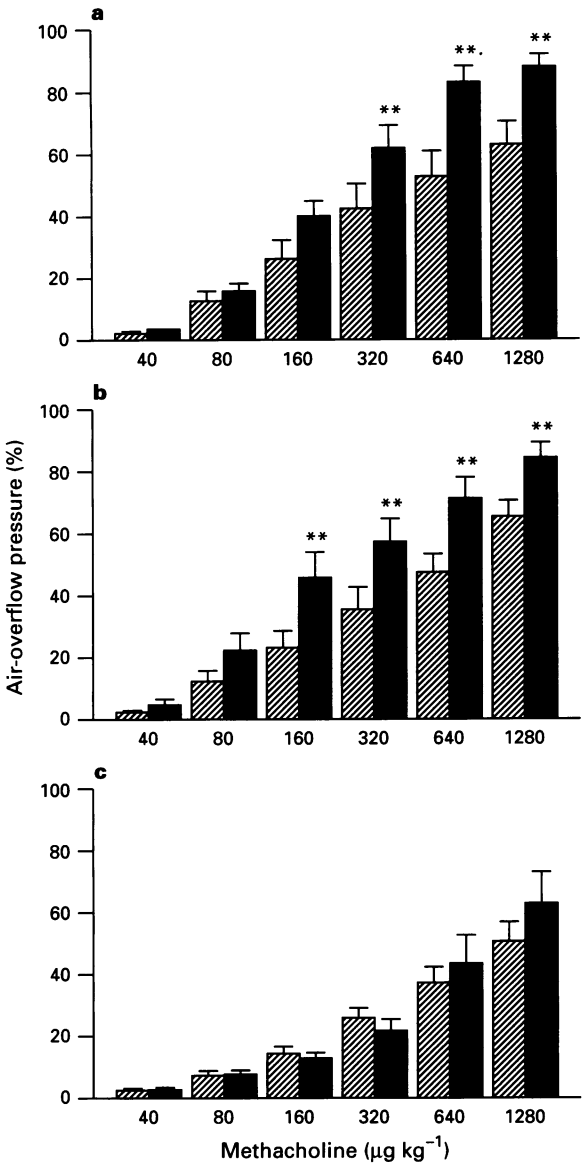

Figure 2 Airway responsiveness after intravenous administration of methacholine in ovalbumin-sensitized mice challenged with ovalbumin (solid columns) or saline (hatched columns) and treated with vehicle (a), metyrapone (b) or dexamethasone (c) 24 h after the last challenge. Results are expressed as arithmetic average ± s.e.mean ($n=6$ per group). **Significantly different from saline-challenged animals ($P<0.01$, Student's t test).

nificant increase in responsiveness to methacholine after ovalbumin challenge was already observed at 160 μg kg^{-1}, whereas in vehicle-treated ovalbumin-challenged animals this was not observed until 320 μg kg^{-1} methacholine. Dexamethasone inhibited the induction of hyperresponsiveness after ovalbumin challenge completely at all dosages of methacholine (Figure 2c). The maximal response at the highest dose of methacholine was decreased by 57% ($P<0.05$) compared to vehicle-treated ovalbumin-challenged mice. Furthermore, no significant differences were observed among the methacholine concentration-response curves of saline-challenged animals from different treatments nor were any significant differences observed in ED_{50} values between saline and ovalbumin-challenged animals of all different treatment groups (data not shown). Ovalbumin-sensitized and challenged animals showed airway hyperresponsiveness after both intravenously administered methacholine (Figure 3a) and 5-HT (Figure 3b) compared to saline-challenged animals.

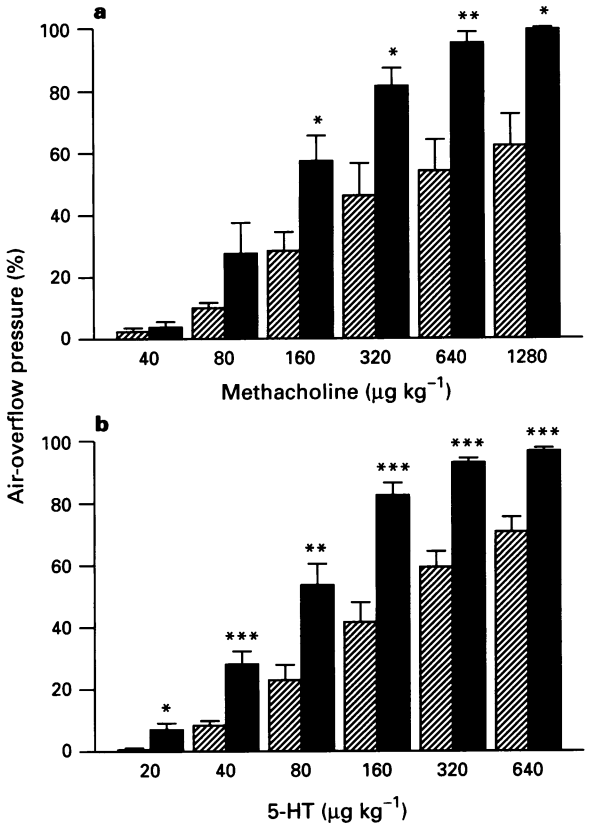

Figure 3 Airway-responsiveness after intravenous administration of methacholine (a) or 5-hydroxytryptamine (5-HT) (b) in ovalbumin-sensitized mice challenged with ovalbumin (solid columns) or saline (hatched columns) 24 h after the last challenge. Results are expressed as arithmetic average ± s.e.mean ($n=6$ per group). *Significantly different from saline-challenged animals ($P<0.05$, Student's t test), **($P<0.01$ Student's t test), ***($P<0.001$, Student's t test).

Bronchoalveolar lavage

Bronchoalveolar lavage of animals was evaluated at both 3 and 24 h after the last challenge. Virtually no eosinophils could be detected in BAL fluid of saline-challenged groups (Table 1). At 3 h and at 24 h after the last ovalbumin challenge a significant migration of eosinophils into the bronchoalveolar lavage was found in vehicle-treated mice (3 h: 5.5×10^3, $P<0.01$; 24 h: 9.1×10^3, $P<0.01$) (Figure 4). Administration of metyrapone potentiated infiltration of eosinophils after ovalbumin challenge at both time-points compared to vehicle-treated ovalbumin-challenged animals (3 h: 17.4×10^3, 24 h: 18.7×10^3, $P<0.01$) (Table 1, Figure 4), whereas dexamethasone treatment completely inhibited eosinophil infiltration (3 h: 0.0, $P<0.01$; 24 h: 0.7×10^3, $P<0.01$) (Table 1, Figure 4). Infiltration of neutrophils and mononuclear cells (lymphocytes, monocytes and macrophages) was not significantly different between ovalbumin and saline-challenged groups, nor were any differences observed between differently treated mice at either time-point (Table 1). Furthermore, no significant differences were observed in total number of cells recovered, except for an increase observed in vehicle-treated animals at 3 h after ovalbumin challenge ($P<0.05$) (Table 1).

Serum ovalbumin-specific immunoglobulin E levels

Levels of ovalbumin (OVA)-specific immunoglobulin E were determined in sera collected at the time of death. A significant increase in the level of OVA-specific IgE was found in all three ovalbumin-challenged groups compared to their

Table 1 Different cell types present in bronchoalveolar lavage

	Total cell numbers ($\times 10^5$)	*Mononuclear cells* ($\times 10^5$)	*Neutrophils* ($\times 10^3$)	*Eosinophils* ($\times 10^3$)
3 h				
Veh/S	2.47±0.25	2.47±0.25	0	0
Veh/O	3.76±0.54 (†)	3.69±0.55	1.06±0.51	5.48±3.8 (*)
Dex/S	3.33±0.59	3.33±0.58	0.40±0.24	0
Dex/O	2.84±0.39	2.81±0.38	2.76±1.66	0 (#)
Met/S	3.23±0.74	3.23±0.74	0.30±0.13	0
Met/O	3.27±0.59	3.04±0.49	4.86±2.93	17.4±9.93 (*)
24 h				
Veh/S	4.29±0.60	4.25±0.60	3.86±1.90	0.20±0.17
Veh/O	4.73±0.32	4.61±0.32	2.36±0.44	9.13±1.67 (*)
Dex/S	4.03±0.40	3.99±0.40	3.83±0.97	0
Dex/O	4.15±0.23	4.13±0.23	1.36±0.64	0.70±0.33 (#)
Met/S	4.35±0.58	4.35±0.58	0.63±0.31	0
Met/O	3.71±0.51	3.50±0.51	2.80±0.51	18.7±2.57 (*)(#)

Total numbers of various cell types in BAL fluid recovered 3 h and 24 h after the last ovalbumin (O) or saline (S) challenge in ovalbumin-sensitized mice treated with vehicle (Veh), dexamethasone (Dex) or metryrapone (Met). Data are expressed as arithmetic average±s.e.mean. †Significantly different from vehicle-treated saline-challenged mice ($P<0.05$, ANOVA); *significantly different from saline-challenged animals ($P<0.05$, ANOVA with Poisson distribution); #significantly different from vehicle-treated ovalbumin-challenged animals ($P<0.05$, ANOVA with Poisson distribution).

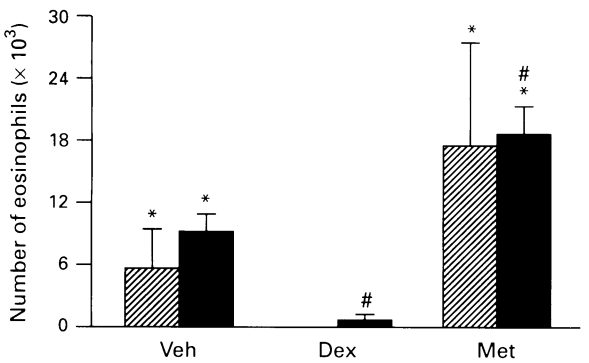

Figure 4 Total numbers of eosinophils recovered at 3 h (hatched columns) and 24 h (solid columns) after the last ovalbumin challenge in BAL fluid of ovalbumin-sensitized, vehicle (Veh), dexamethasone (Dex) or metyrapone (Met) treated mice. Virtually no eosinophils were present in all saline-challenged animals (Table 1). Results are expressed as arithmetic average±s.e.mean ($n=6$ per group). *Significantly different from vehicle-treated saline-challenged animals ($P<0.05$, ANOVA with Poisson distribution). # Significantly different from vehicle-treated ovalbumin-challenged animals ($P<0.05$, ANOVA with Poisson distribution).

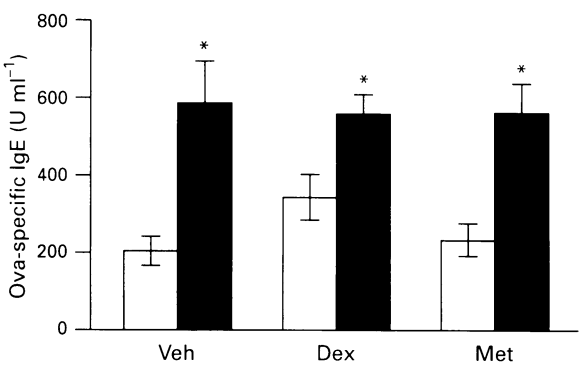

Figure 5 Ovalbumin-specific Immunoglobulin E (IgE) levels measured by ELISA in serum after saline challenge (open columns) or after ovalbumin challenge (solid columns) in ovalbumin-sensitized mice after vehicle (Veh), dexamethasone (Dex) or metyrapone (Met) treatment. Serum was collected after the last challenge. Results are expressed as arithmetic average±s.e.mean (n is at least 5 per group). *Significantly different from immunoglobulin E level in saline-challenged animals ($P<0.05$, Student's t test).

saline-challenged controls ($P<0.05$) (Figure 5). No significant differences in serum OVA-IgE titres were found among either saline or ovalbumin-challenged animals treated with vehicle, metyrapone or dexamethasone.

Plasma corticosterone levels

Plasma corticosterone levels were determined in samples collected from vehicle and metyrapone-treated animals. In vehicle-treated ovalbumin-challenged mice a plasma level of 56.8±11.2 ng ml^{-1} was found (Figure 6), which was not significantly different from plasma corticosterone levels found in saline-challenge animals (74.2±13.0 ng ml^{-1}). Metyrapone treatment resulted in a significant reduction ($P<0.001$) of plasma corticosterone levels in both ovalbumin and saline-challenged mice (4.6±0.8 ng ml^{-1}; 3.6±1.1 ng ml^{-1}, respectively) indicating that metyrapone was successful in inhibiting the 11β-hydroxylase-mediated formation of corticosterone.

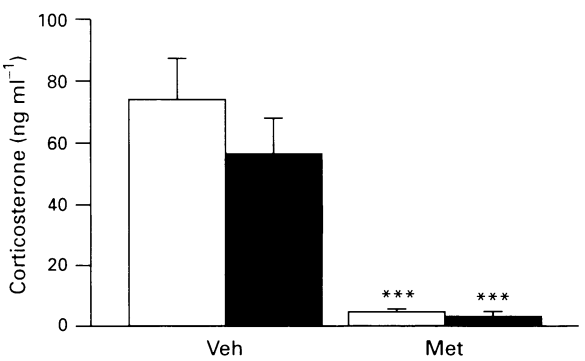

Figure 6 Corticosterone levels were measured by use of a ^{125}I radioimmunoassay in plasma from vehicle-treated (Veh) or metyrapone-treated animals (Met). Plasma was collected after the last challenge. No differences were observed between either saline (open columns) or ovalbumin-challenged (solid columns) animals. Results are expressed as arithmetic average±s.e.mean ($n=6$ per group). ***Significantly different from corticosterone level in vehicle-treated animals ($P<0.001$, Student's t test).

Discussion

Previously, it was demonstrated that the ovalbumin sensitization and challenge procedure described here induced several asthma related phenomena. After sensitization OVA-specific immunoglobulin E serum levels could be detected (Hessel *et al.*, 1995a) and when sensitized mice were challenged with ovalbumin, eosinophil infiltration in both BAL and lung tissue was observed as well as an increase in responsiveness of isolated tracheae to methacholine (Hessel *et al.*, 1994). Furthermore $CD4^+$ T-cells were increased in BAL and the Th2-type cytokines interleukin-4 and interleukin-5 were detected in BAL fluid (unpublished observations).

In this study the role of both exogenous as well as endogenous corticosteroids on the development of both *in vivo* and *in vitro* airway hyperresponsiveness and inflammatory cell infiltration was investigated. Dexamethasone, when given during the challenge period prevented both *in vitro* and *in vivo* hyperresponsiveness to methacholine as well as infiltration of eosinophils into BAL fluid at 3 and 24 h after the last ovalbumin challenge. In contrast to these observations, ovalbumin-challenged mice treated with metyrapone showed a potentiation of *in vitro* hyperresponsiveness compared to vehicle-treated ovalbumin-challenged mice. Additionally, significant hyperresponsiveness *in vivo* was observed at lower doses of methacholine, indicating an increase in sensitivity to methacholine. Compared to vehicle-treated ovalbumin-challenged animals, eosinophil infiltration was also significantly increased after metyrapone treatment. Plasma corticosterone measurements revealed that inhibition of 11β-hydroxylase by metyrapone was successful since plasma corticosterone levels in these animals were dramatically decreased compared to vehicle-treated mice. Furthermore, we demonstrated in this study that the observed *in vivo* hyperresponsiveness is non-specific, since ovalbumin-sensitized and challenged mice were hyperresponsive for both methacholine and 5-HT compared to saline-challenged animals.

From our study the conclusion can be drawn that an exogenously applied corticosteroid, such as dexamethasone, can inhibit both airway hyperresponsiveness and eosinophil infiltration into the airways. Interestingly, we also demonstrated in this study that endogenously produced corticosteroids play an inhibitory role in the development of airway hyperresponsiveness and eosinophilia in our model, since both phenomena could be enhanced by depletion of endogenous corticosterone.

In rat models of allergic asthma the role of corticosteroids has been investigated. Renzi *et al.* (1993) showed that dexamethasone treatment inhibited inflammatory cell infiltration but not airway hyperresponsiveness in their rat model for ovalbumin-induced asthma. In contrast, Elwood *et al.* (1992b) demonstrated that rats sensitized and challenged with ovalbumin revealed no airway hyperresponsiveness to methacholine and allergen-induced inflammatory cell infiltration after dexamethasone treatment. In agreement with our findings, Kung *et al.* (1994) also observed a reduction in ovalbumin-induced infiltration of eosinophils in bronchoalveolar lavage fluid after corticosteroid treatment in their mouse model for allergic pulmonary inflammation.

The exact mechanisms by which corticosteroids can influence the development of both bronchial hyperresponsiveness and eosinophil infiltration remain to be elucidated, but it is very tempting to speculate that the main effect of corticosteroids in allergic reactions is on cytokine production by inflammatory cells. This speculation is sustained by several other investigators who demonstrated that corticosteroids such as dexamethasone can affect cytokine production of Th2-type $CD4^+$ cells both in *in vitro* cell cultures and in asthmatic patients (Schmidt *et al.*, 1994; Mori *et al.*, 1994). Besides Th2-type cytokines, corticosteroids can also inhibit production of Th1-type cytokines such as IL-2 and IFNγ (see Nijkamp & Van Oosterhout, 1993). Previously, we demonstrated that both Th1 and Th2-type cytokines are important in the development of asthma related phenomena in our model (Hessel *et al.*, 1996). Therefore, the inhibitory effect of corticosteroids on eosinophil infiltration and airway hyperresponsiveness may be explained by inhibition of cytokine production.

Several other explanations can be postulated to explain the inhibition of both airway hyperresponsiveness and eosinophilia besides a direct effect of corticosteroids on cytokine production. First, both IL-1 production and antigen presentation by mononuclear cells can be inhibited by corticosteroids (Snyder & Unanue, 1982), which may be even more important for the inhibition of lymphocyte activation and cytokine secretion *in vivo*. Second, phospholipase A_2 activity could be inhibited in lung tissue (Nijkamp *et al.*, 1976). In this way both the cyclo-oxygenase and the lipoxygenase pathways are inhibited. As a result the synthesis of all arachidonic metabolites, including the leukotrienes C_4 and D_4, is reduced. These mediators have bronchoconstrictive properties, can cause airway hyperresponsiveness and are chemotactic for inflammatory cells (see Nijkamp & Van Oosterhout, 1993).

In our study enhancement of airway hyperresponsiveness and eosinophil infiltration was observed after depletion of endogenous corticosteroids by metyrapone treatment. In the literature, endogenous corticosteroid production has been described as enhanced after activation of the immune system (Besedovsky *et al.*, 1981). This increase in endogenous corticosteroid production probably serves as a physiological negative feed-back mechanism, since several studies in experimental animal models have demonstrated down-regulatory actions of endogenous corticosteroids on immune parameters such as inflammatory reactions (Richards *et al.*, 1992; Peers *et al.*, 1993; Fornhem *et al.*, 1995). To the best of our knowledge, we are the first to show that depletion of endogenous corticosteroids by metyrapone leads to a significant increase in eosinophil infiltration in bronchoalveolar lavage fluid accompanied by a potentiation of airway hyperresponsiveness.

No changes were found in OVA-specific immunoglobulin E production after ovalbumin challenge in both dexamethasone and metyrapone-treated animals compared to vehicle-treated ovalbumin-challenged mice, suggesting that both exogenous and endogenous corticosteroids have no effect on immunoglobulin E production during the challenge period. This is in agreement with the commonly known fact that the humoral immune response is steroid-resistant compared to cellular-mediated immunity (see Nijkamp & Van Oosterhout, 1993). Furthermore, it is known that after second contact with antigen, immunoglobulin E production by B-cells is less dependent on stimulation by $CD4^+$ T-cell-derived cytokines and cognate B-T-cell contact (Van Ommen *et al.*, 1994). Thus, although corticosteroids may have inhibited cytokine production by T-cells in our study, the immunoglobulin E production induced by ovalbumin challenges in already sensitized mice appears to be resistant to both endogenous and exogenous corticosteroids.

In summary, it can be concluded that, similar to patients with allergic asthma, exogenously applied corticosteroids can inhibit eosinophil infiltration as well as airway hyperresponsiveness in this murine model of allergic asthma. In addition, endogenous corticosteroids have a down-regulatory role since both eosinophilia and hyperresponsiveness were increased after inhibition of endogenous corticosteroid synthesis. It can be speculated that inhibition of both eosinophilia and airway hyperresponsiveness by corticosteroids is caused by a decrease in cytokine production by T-cells, a commonly known upstream steroid-sensitive cell in the pathway leading to allergic inflammatory reactions and airway hyperresponsiveness. Furthermore, our data imply that the model described in this study could very well be used in the screening of new steroid-like drugs used for the treatment of asthmatics as well as for studying the precise mechanism of steroid action.

The authors gratefully acknowledge L.P. Kegler, and P.J. van Schaaik from the animal housing department, A. Muis for her

technical assistance in some experiments, W.E. van der Wal for measurement of plasma corticosterone levels and Dr P. Westers for help with the statistical analyses. Financial support was obtained for I.VA. from The Netherlands Asthma Foundation (AF93.63), and for B.VE. from Genentech Inc. (South San Francisco, CA, U.S.A.).

References

BEASLY, R., ROCHE, W.R., ROBERTS, J.A. & HOLGATE, S.T. (1989). Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.*, **139,** 806–817.

BESEDOVSKY, H.O., DEL REY, A. & SORKIN, E. (1981). Lymphokine-containing supernatants from Con A-stimulated cells increase corticosterone blood levels. *J. Immunol.*, **126,** 385–387.

CARTIER, A., THOMSON, N.C., FRITH, P.A., ROBERTS, R. & HARGREAVE, F.E. (1982). Allergen-induced increase in bronchial responsiveness to histamine: relationship to the late asthmatic response and change in airway caliber. *J. Allergy Clin. Immunol.*, **70,** 170–177.

CLARK, T.J.H. & HETZEL, M.R. (1977). Diurnal variation of asthma. *Br. J. Dis. Chest.*, **71,** 87–92.

DJUKANOVIC, R., ROCHE, W.R., WILSON, J.W., BEASLY, C.R.W., TWENTYMAN, O.P., HOWARTH, P.H. & HOLGATE, S.T. (1990). Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.*, **142,** 434–457.

DUNNIL, M.S. (1978). The pathology of asthma. In *Allergy Principles and Practice (2nd edition)*. ed. Middleton, E. Jr., Reed, C.E. & Ellis, E.F. pp. 678–686. St. Louis, MO: C.V. Mosby Co.

ELWOOD, W., BARNES, P.J. & CHUNG, K.F. (1992a). Airway hyperresponsiveness is associated with inflammatory cell infiltration in allergic Brown-Norway rats. *Int. Arch. Allergy Immunol.*, **99,** 91–97.

ELWOOD, W., LÖTVALL, J.O., BARNES, P.J. & CHUNG, K.F. (1992b). Effect of dexamethasone and cyclosporin A on allergen-induced airway hyperresponsiveness and inflammatory cell responses in sensitized Brown-Norway rats. *Am. Rev. Respir. Dis.*, **145,** 1289–1294.

FORNHEM, C., LUNDBERG, J.M. & ALVING, K. (1995). Allergen-induced late-phase airways obstruction in the pig: the role of endogenous cortisol. *Eur. Respir. J.*, **8,** 928–937.

GARSSEN, J., VAN LOVEREN, H., VAN DER VLIET, H. & NIJKAMP, F.P. (1990). An isometric method to study respiratory smooth muscle responses in mice. *J. Pharm. Methods*, **24,** 209–217.

HESSEL, E.M., VAN OOSTERHOUT, A.J.M., HOFSTRA, C.L., GARSSEN, J., VAN LOVEREN, H., SAVELKOUL, H.F.J. & NIJKAMP, F.P. (1994). Repeated ovalbumin inhalation causes bronchial hyperresponsiveness and eosinophil infiltration in sensitized mice. *Am. J. Respir. Crit. Care Med.*, **149,** A754.

HESSEL, E.M., VAN OOSTERHOUT, A.J.M., HOFSTRA, C.L., DE BIE, J.J., GARSSEN, J., VAN LOVEREN, H., VERHEYEN, A.K.C.P., SAVELKOUL, H.F.J. & NIJKAMP, F.P. (1995). Bronchoconstriction and airway hyperresponsiveness after ovalbumin inhalation in sensitized mice. *Eur. J. Pharmacol.*, **293,** 401–412.

HESSEL, E.M., VAN OOSTERHOUT, A.J.M., VAN ARK, I., VAN ESCH, B., HOFMAN, G., SAVELKOUL, H.F.J. & NIJKAMP, F.P. (1996). Development of airway hyperresponsiveness is dependent on IFNγ and independent of eosinophil infiltration. . *Am. J. Respir. Cell Mol. Biol.* (in press).

IIJIMA, H., ISHII, M., YAMAUCHI, K., CHAO, C-L., KIMURA, K., SHIMURA, S., SHINDOH, Y., INOUE, H., MUE, S. & TAKISHIMA, T. (1987). Bronchoalveolar lavage and histologic characterization of late asthmatic response in guinea pigs. *Am. Rev. Respir. Dis.*, **136,** 922–929.

KALLENBACH, J.M., PANZ, V.R., JOFFE, B.I., JANKELOW, D., ANDERSON, R., HAITAS, B. & SEFTEL, H.C. (1988). Nocturnal events related to "morning dipping" in bronchial asthma. *Chest*, **93,** 751–757.

KUNG, T.T., JONES, H., ADAMS III, G.K., UMLAND, S.P., KREUTNER, W., EGAN, R.W., CHAPMAN, R.W. & WATNICK, A.S. (1994). Characterization of a murine model of allergic pulmonary inflammation. *Int. Arch. Allergy Immunol.*, **105,** 83–90.

MACKAY, T.W., WALLACE, W.A.H., HOWIE, S.E.M., BROWN, P.H., GREENING, A.P., CHURCH, M.K. & DOUGLAS, N.J. (1994). Role of inflammation in nocturnal asthma. *Thorax*, **49,** 257–262.

MORI, A., SUKO, M., NISHIZAKI, Y., KAMINUMA, O., MATSUZAKI, G., ITO, K., ETOH, T., NAKAGAWA, H., TSURUOKA, N. & OKUDAIRA, H. (1994). Regulation of interleukin-5 production by peripheral blood mononuclear cells from atopic patients with FK506, cyclosporin A and glucocorticoid. *Int. Arch. Allergy Immunol.*, **104,** 32–35.

NADEL, J.A. & SHEPPARD, D. (1985). Mechanisms of bronchial hyperreactivity in asthma. In *Bronchial Asthma: Mechanisms and Therapeutics*. ed. Weiss, E.B., Segal, M.S. & Stein, M., pp. 30–36. Boston: Little Brown.

NIJKAMP, F.P., FLOWER, R.J., MONCADA, S. & VANE, J.R. (1976). Partial purification of rabbit aorta contracting substance-releasing factor and inhibition of its activity by anti-inflammatory steroids. *Nature*, **263,** 479–482.

NIJKAMP, F.P. & VAN OOSTERHOUT, A.J.M. (1993). Mechanisms of action of glucocorticoids in the treatment of asthma. In *Topics in Pharmaceutical Sciences*. ed. Crommelin, D.J.A., Midha, K.K. & Nagai, T., pp. 77–88. Stuttgart: Medpharm Scientific Publishers.

O'BYRNE, P.M., DOLOVICH, J. & HARGREAVE, F.E. (1987). Late asthmatic responses. *Am. Rev. Respir. Dis.*, **136,** 740–751.

PEERS, S.H., DUNCAN, G.S. & FLOWER, R.J. (1993). Development of specific antibody and *in vivo* response to antigen in different rat strains: effect of dexamethasone and importance of endogenous corticosteroids. *Agents Actions*, **39,** 174–181.

RAEBURN, D., UNDERWOOD, S.L. & VILAMIL, M.E. (1992). Techniques for drug delivery to the airways, and the assessment of lung function in animal models. *J. Pharmacol. Toxicol. Methods*, **27,** 143–159.

RENZI, P.M., OLIVENSTEIN, R. & MARTIN, J.G. (1993). Effect of dexamethasone on airway inflammation and responsiveness after antigen challenge of the rat. *Am. Rev. Respir. Dis.*, **148,** 932–939.

RICHARDS, I.M., GRIFFIN, R.L., SHIELDS, S.K., REID, M.S. & FIDLER, S.F. (1992). Chasing the elusive animal model of late-phase bronchoconstriction: studies in dogs, guinea pigs and rats. *Agents Actions*, **37,** 178–180.

RICHARDS, I.M., SHIELDS, S.K., GRIFFIN, R.L. & DUNN, C.J. (1990). A novel model of antigen-induced lung eosinophilia in Brown-Norway rats: Effect of methylprednisolone. *Eur. J. Pharmacol.*, **183,** 1191–1192.

SCHMIDT, J., FLEIßNER, S., HEIMANN-WEITSCHAT, I., LINDSTAEDT, R. & SZELENYI, I. (1994). The effect of different corticosteroids and cyclosporin A on interleukin-4 and interleukin-5 release from murine T_H2-type T-cells. *Eur. J. Pharmacol.*, **260,** 247–250.

SNYDER, D.S. & UNANUE, E.M. (1982). Corticosteroids inhibit murine macrophage Ia expression and interleukin 1 production. *J. Immunol.*, **129,** 1803–1805.

VAN OMMEN, R., VREDENDAAL, A.E.C.M. & SAVELKOUL, H.F.J. (1994). Secondary IgE responses in vivo are predominantly generated via γ1ε-double positive B cells. *Scand. J. Immunol.*, **40,** 491–501.

WEITZMAN, E.D., FUKUSHIMA, D., NOGEIRE, C., ROFFWARG, H., GALAGHER, F. & HELLMAN, L. (1971). Twenty-four hour pattern of the episodic secretion of cortisol in normal subjects. *J. Clin. Endocrinol. Metab.*, **33,** 14–22.

(Received August 27, 1996
Accepted September 6, 1996)